A-Form of Cytoplasmic Domain of nARIA (CRD-Neuregulin) And Uses Thereof

5   This application incorporates by reference the entire disclosures of U.S. Provisional Application No.60/003,380, filed September 7, 1995 and U.S. patent application Serial No. 08/697,954, filed September 4, 1996.

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15   ## Background of the Invention
Throughout this application, various publications are referenced by author and date, *and by arabic numbers in superscript*. Full citations for these publications may be found listed ~~alphabetically~~ at the end of the specification immediately preceding the claims. The
20   disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

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The development and differentiation of embryonic neurons culminates in synapse formation. Neuronal development is an intricate process that involves a cascade of inductive interactions between a neuron and the pre- and postsynaptic
30   partners of that neuron. These highly regulated events are important for the establishment of reliable, yet plastic, synaptic formation and transmission. Correct expression of an array of transmitter-gated channels by neurons is clearly essential to synaptic differentiation, and yet the
35   developmental regulation of this process is poorly understood. In fact, despite overwhelming advances in

probing the molecular and biophysical details of ion channels gated by gamma-amino butyric acid (GABA), glycine, glutamate and acetylcholine (ACh) (Betz, 1990; Deneris et al., 1991; McGehee et al., 1995; Role, 1992; Sargent, 1993)

5 the corresponding embryonic versions of these receptors have evaded analysis. Characterization of the biophysical properties of ligand-gated channels in developing neurons and description of their evolution to the mature receptor profile is limited (Brussard et al., 1994; Moss and Role,

10 1993; Margiotta and Gurantz, 1989). Furthermore, little is known about the mechanism of these changes.

The study of embryonic ligand-gated channels and subsequent modifications of their functional profile during neural

15 development is difficult. Receptor expression prior to synaptogenesis is at a low level. Synapse formation is not synchronous. In the few cases studied, the developmental changes in receptor function are vast (Berg et al., 1989; Engisch and Fischbach, 1992; Arenella et al, 1993; Deneris

20 et al, 1991; McGehee and Role, 1995; Role, 1992; Sargent, 1993). In the establishment of mature synapses, profound alterations in the expression profile of neuronal ligand-gated channels occur. In addition to these changes in expression levels, changes in the cellular distribution, the

25 subunit composition and the biophysical and pharmacological properties occur as well (Margiotta and Gurantz, 1989; Moss and Role, 1993; Moss et al., 1989; Devay et al, 1994; Arenella et al, 1993; Jacob, 1991; Mandelzys et al, 1994; Smith et al, 1983; Vernallis et al, 1993). The interactions

30 between presynaptic and target neurons may play a large role in the extrinsic influences which are believed to modify receptor function throughout development. The mechanism of receptor development remains unclear, however, presynaptic input, target cell regulation, synaptic activity or

35 molecular signals independent of transmission may be

involved.

Diversity of neuronal nicotinic receptors

One important feature of neuronal ligand-gated channels,
5   nicotinic acetylcholine receptors (nAChRs) in particular, is
the diversity of component subunits and the resultant
diversity in channel subtypes (Boulter et al, 1986; Conroy
et al., 1992; Grynkiewicz et al., 1985; Lindstrom et al.,
1990; Luetje and Patrick, 1991; McGehee and Role, 1995;
10   Papke and Heinemann, 1991; Ramirez-Latorre et al.,
submitted; Role, 1992). Neuronal nAChRs were the first of
the ligand-gated ion channels studied to display this degree
of structural and functional complexity. Although nAChRs
comprise only two distinct subunit types, there are multiple
15   homologous forms of each subunit encoding gene. There are
8 neuronal "α" subunit genes ($\alpha1$-$\alpha8$) and 3 neuronal "ß"
subunit genes (ß2-ß4) cloned to date (Boulter et al., 1986;
Heinemann et al., 1990; Nef et al., 1988; Seguela et al.,
1993; Wada et al., 1989). With this array as a starting
20   point, there could be more than 10 varieties of pentameric
nAChR complexes (McGehee et al., 1995 and Role, 1992).
Study of native nAChRs indicates that the actual number of
subunit combinations is less than theory would predict.
Biochemical, immunochemical, and antisense deletion
25   experiments to identify native compositions of nAChRs
demonstrate that relatively few subunit combinations are
likely to be found in native nAChRs. For example, the
nAChRs expressed by autonomic and habenula neurons have been
studied in detail (Brussard et al., 1994; Devay et al.,
30   1994; Listerud et al., 1991; Clarke et al., 1986) and
provide specific examples of the subunit composition of each
nAChR channel subtype expressed. In view of the documented
evolution of these neuronal nAChR channels during embryonic
development, and the array of molecular and biophysical
35   tools available to study these channels in detail, an

understanding of the developmental regulation of nAChR subunit and channel subtype diversity may be close at hand. Numerous studies implicate the interaction during the formation of synaptic connections between the presynaptic and postsynaptic cells in the development of mature neuronal receptors (Arenella et al., 1993; Boyd et al., 1988; Brussaard et al., 1994b; Brussaard et al., 1994; Devay (in preparation; Devay et al., 1994; Gardette, et al., 1991; Jacob 1991; Levey et al., 1994; Mandelzys et al., 1994; Moss et al., 1989).

Regulation of neuronal phenotype during development: contribution of target interactions.

Neuronal differentiation is induced by the interaction of developing neurons with target cells. One example is that of the evolution of transmitter phenotype in a special class of sympathetic neurons that evolve from an adrenergic to a cholinergic phenotype in the course of normal development. Although early on, these neurons synthesize, package and release catecholamines, the formation of synapses with the target sweat glands is accompanied by a change in transmitter expression that ultimately produces a mature cholinergic phenotype. This change in transmitter expression requires both pre- and postsynaptic signals. Thus, catecholamine release from the embryonic neuron is required to induce the release of a cell differentiation factor\leukemia inhibitory factor (CDF/LIF)-like factor called sweat gland factor (SGF) from the presumptive sweat glands. SGF, released via activation of target adrenergic receptors, interacts, with specific receptors on the innervating neuron. SGF induces the cellular machinery required for ACh synthesis and release in the presynaptic neuron. Thus, the attainment of a mature transmitter phenotype is regulated by both synaptic activity and target derived signals, offering an explanation for how the

innervation of target tissues regulates nAChR expression in CNS and PNS neurons.

This example is one of many implicating target-derived factors in the control of neuronal survival, proliferation, differentiation, migration, and neurite outgrowth. Although there are many factors that could mediate target effects on neuronal differentiation, the expression patterns and biological activities of factors identified to date identify a few candidates for proposed studies of nAChR regulation. (1) Ciliatory neurotropic factor (CNTF) mimics the effect of SGF in inducing a cholinergic phenotype. It has also been shown to promote differentiation of sympathetic precursor cells and likely participates in target-induced changes in nAChR expression. CNTF is expressed in numerous sympathetic targets including smooth muscle and kidney. (2) Activin and related members of the Transforming Growth Factor ß (TGFß) family, can also regulate the differentiation of the transmitter phenotype in autonomic neurons. These factors are expressed in sympathetic targets such as smooth muscle, sweat glands etc. (3) CDF/LIF mimics SGF by inducing a cholinergic phenotype. This factor is secreted by smooth muscle and heart muscle. Although less is known about either the activity or the distribution of these factors in the central nervous system (CNS), it is likely that CNS and peripheral nervous system (PNS) neurons may be regulated by similar signaling molecules.

Regulation of receptor synthesis and distribution in muscle: contribution of presynaptic signals

Classical studies of Fischbach, Cohen, and McMahan of the nerve-muscle junction demonstrate that the incoming motor nerve is a potent regulator of muscle-nAChRs. Prior to innervation, muscle expresses an embryonic form of nAChR which is diffusely distributed over the cell surface. The

expression of the muscle-nAChR is eventually downregulated to a diffuse distribution. Elimination of muscle-nAChRs by innervation is accompanied by an increase in local synthesis, insertion and formation of high-density clusters

5   of muscle-nAChR at the synaptic site. At later stages of synaptic development, there are marked changes in the biological properties of muscle-nAChR channels due to alterations in subunit gene expression. This produces "adult" type muscle-nAChR complexes of distinct subunit

10   composition. Molecular signals that are believed to mediate these changes in muscle-nAChR distribution and synthesis have been identified and cloned, namely, agrin and AChR Inducing Activity (ARIA). Recombinant agrin alters the distribution of pre-existent muscle-nAChRs with no effect on

15   synthesis or insertion of new receptors. In contrast, recombinant ARIA induces muscle-nAChR subunit gene expression, increasing the rate of appearance of new surface receptors from 3-5%/hr to 10-20%/hr.

20   It is possible that there are common regulatory mechanisms between nAChR and muscle-nAChR. It is believed that nAChRs on both CNS and PNS neurons evolve from low density and diffuse distribution to clustered and highly dense synaptic patches following innervation. Finally, like muscle-nAChRs,

25   there are marked changes in the biophysical properties of nAChRs during development and presynaptic input may induce some of these changes, e.g., channel conductance and opening frequency.

30   Despite the essential role of ligand-gated ion channels in synaptic transmission between neurons, little is known about changes in their expression, function, distribution and subunit composition during neural development. Nicotine-induced enhancement of acquisition and consolidation of

35   short term memories is believed to be mediated by

presynaptic nAChR activation since this activation facilitates a broad array of CNS synapses. In view of the impact that developmental changes in nAChRs have on neuronal excitability, synaptic efficacy and synaptic plasticity,
5 studies of the regulatory controls of nAChR expression are essential. One avenue of study focuses upon the proteinaceous factors that appear to modulate receptor gene expression. One factor previously identified and cloned is heregulin (See Vandlen and Holmes, U.S. Patent No.
10 5,367,060).

To date, 10 different proteins that result from alternative splicing of the heregulin gene have been described. Among these are the growth factors neu differentiation factor
15 (NDF) (Wen et al., 1992), glial growth factor (GGF) (Marchionni et al., 1993), ARIA (Falls et al, 1993; Fischbach et al., 1994), and the heregulin isoforms (Holmes, 1992; Wen et al., 1994). The reported isoforms are principally membrane bound proteins which can be solubilized
20 by proteolysis. The extracellular domains of these proteins consist of an N-terminal domain followed by an immunoglobulin-like (Ig-like) domain, a linker region, an EGF-like domain, and a second linker region (Figure 5). These proteins are ligands for the epidermal growth factor
25 (EGF) family of receptor tyrosine kinases. Binding of the ligand to the EGF receptor family members erbB3/HER3 or HER4 results in activation of the tyrosine kinase activity of the receptor. Other family members can be activated by trans-phosphorylation via the activated members.
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Other members of the heregulin/NDF/ARIA family have been described in previous patent publications. PCT International Publication No. WO 94/08007, published April 14, 1994, entitled "Trophic factor having ion channel-
35 inducing activity in neuronal cells" describes neurotrophic

factors designated as ARIA, which are able to induce the formation of ion channels. This publication also shows how ARIA is associated with both nervous tissue and skeletal muscle. ARIA has an Ig-like domain and an EGF-like domain
5   in the extracellular region. U. S. Patent No. 5,367,060, issued November 22, 1994, entitled "Structure, production and use of heregulin", from U.S. Serial No. 847,743, filed March 6, 1992 by Richard L. Vandlen and William E. Holmes, discloses a polypeptide with a binding affinity for the
10   $p185^{HER2}$ receptor. Vandlen and Holmes also disclose purification methods required to isolate heregulin and uses of the heregulins and antibodies specific to the heregulins as therapeutic agents. This polypeptide is related to but distinct from the ARIA protein.

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Summary of the Invention

This invention provides an assay for diagnosing whether a subject has or is predisposed to developing a neoplastic disease which comprises: a) obtaining a biological sample

5   from the subject; b) contacting the sample with an agent that detects the presence of an extracellular domain of nARIA (CRD-neuregulin) or an isoform thereof; c) measuring the amount of agent bound by the sample; d) comparing the amount of agent bound measured in step c) with the ~~the~~

10   amount of agent bound by a standard normal sample, a higher amount bound by the sample from the subject being indicative of the subject having or being predisposed to developing a neoplastic disease. One embodiment of this invention is a method for maintaining synaptic connections between a neuron

15   and a target cell comprising contacting the target cell with an nARIA polypeptide or a nucleic acid molecule encoding nARIA in an amount sufficient to induce the formation of a synaptic junction.

Brief Description of the Figures

Figures 1A-1C: Nucleotide sequence of chicken nARIA (SEQ ID NO: 1).

5   A nucleic acid sequence encoding a splice variant from the
heregulin gene is shown. This sequence is a compilation of
the sequences derived from the ExoIII deletion series on the
pBluescript II KS (+) subclone of phage #3 from a chick E13
total brain cDNA library screened with a rat pro-heregulin
10   beta 1 probe generated by PCR amplification. The sequence
was determined using the M13 reverse primer. The length is
3212 bases. The break in homology to the ARIA sequence
occurs at a known splice site. The nucleotide sequences
from base pair number 1293 downstream to the poly-A tail of
15   the nARIA clone are identical to ARIA. The sequences
upstream from base pair number 1293 encode a unique splice
variant (i.e. bp 1293-bp 3212), nARIA.

Figure 2: Amino acid sequence of chicken nARIA (SEQ ID NO: 2).

20   An amino acid sequence encoding a splice variant from the
heregulin gene is shown. This sequence is a compilation of
the sequences derived from the ExoIII deletion series on the
pBluescript II KS (+) subclone of phage #3 from a chick E13
25   total brain cDNA library screened with a rat pro-heregulin
beta 1 probe generated by PCR amplification. The sequence
was arrived at using the M13 reverse primer. The length is
1070 amino acids. The asteriks denote unclear results at
these stop codons.

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Figure 3: Nucleotide sequence of the unique portion of the human nARIA gene (SEQ ID NO: 3).

A nucleic acid sequence encoding the human nARIA (hnARIA) a
splice variant from the heregulin gene, nARIA is shown. The
35   product was subcloned into pBluescript II KS (+) the 5' end

of the transcript is at the M13 end of the multiple cloning site (MCS). The length is 1351 bases. The unique portion of nARIA spans from base 93 to base 758.

5 **Figure 4: Amino Acid sequence of the unique portion of the human nARIA protein (SEQ ID NO: 4).**

An amino acid sequence encoding the unique portion of the human nARIA is shown. The product was subcloned into pBluescript II KS (+) the 5' end of the transcript is at the
10 M13 end of the MCS. The length is 449 amino acids.

Figures 5A-5B: Comparison of gene structure of nARIA with ARIA

(A) Comparison of the gene structure of the chicken nARIA
15 cDNA with ARIA human nARIA, heregulin, and NDF. (B) Comparison of the exon structure of splice variants of the ARIA/NDF/Heregulin gene. The sequence we cloned (nARIA) has a unique N terminal domain devoid of Ig-like repeats common to other ARIA variants.

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Figure 6: Stage E13 multiple tissue Northern blot.

Multiple tissue Northern blots were screened with probes specific for unique domains of nARIA and were compared with those probed with an ARIA specific probe. The ARIA probe
25 indicates that this form is present in skeletal muscle (pectoral muscle) whereas expression of nARIA is restricted to nervous tissue.

Figure 7: Developmental Northern analysis of ARIA and nARIA
30 **in the chick hindbrain and cerebellum**

Northern blot analysis was performed on RNA samples from chick embryonic stages E4 through E16. Oligonucleotide probe specific for either nARIA or ARIA were used as probes. (H-hindbrain; C-cerebellum.)

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Figures 8A-8B: RT-PCR analysis of the developmental expression pattern of nARIA in the chick brain compared with ARIA.

(A) The developmental expression pattern of nARIA and ARIA
5   in chick brain as detected by RT-PCR is shown. (B) Relative quantification of ARIA and nARIA mRNA levels normalized with actin mRNA levels is shown.

Figures 9A, 9B and 9C: nARIA and ARIA expression in the
10   **developing chick spinal cord as detected by PCR and Northern hybridization analysis.**

(A) Northern blot of ARIA (top) and nARIA (bottom). All probes and primers are directed against sequences specific to nARIA and/or ARIA. Northern analysis indicates the mRNA
15   of nARIA is detectable by E3 and robust by E4 whereas initiation of ARIA expression occurs later (E6-8) and is detectable at E6, but not robust until E8. (B) PCR detection of mRNA expression of ARIA and nARIA in developing chick spinal cord. Note specificity of primers tested on
20   full length cDNAs (NA, A) (-RT: no reverse transcriptase reaction; NA - nARIA positive control; A - ARIA positive control). (C) Comparison of nARIA and ARIA mRNA levels relative to actin.

25   **Figures 10A-10D:** *In situ* **hybridization of chick ED5 trunk cross-section of neural tissue with probes specific for nARIA and ARIA.**

The probe specific for nARIA contains the Cys-rich domain and the probe specific for ARIA contains the Ig domain.
30   Different patterns of expression are observed. A positive signal is observed in the presumptive preganglionic neurons with the nARIA probe but not with the ARIA probe. (A-D) ED5 trunk cross section. (A,C) nARIA specific probe. (B,D) ARIA specific probe.

Figures 11A, 11B and 11C: nARIA induces tyrosine phosphorylation.

(A) Phosphorylation of MCF7 cell line demonstrating activity of both recombinant ARIA and nARIA as ligands for tyrosine kinase-linked receptors. Dose (A) and time (C) dependence of ARIA (A) and nARIA (A,C) phosphorylations in MCF-7 cells. (B) Comparison of recombinant nARIA and ARIA tyrosine phosphorylation of E9 lumbar sympathetic ganglia (LSG).

Figure 12: Anti Phosphotyrosine Western Blot of MDA-MB-453 cells treated with conditioned media.

Media conditioned by COS1 or HEK293 cells transiently transfected with the nARIA clone (sense configuration) activates tyrosine kinase activity in the breast tumor cell lines MCF7 or MDA-MB-453 above the basal levels of tyrosine kinase activity (antisense configuration). The levels of phosphorylation of EGFR family members relative to one another was different between ARIA and nARIA.

Figures 13A-13D: Electrophysiological assay of transmitter gated macroscopic currents.

(A,C) ACh-evoked current. (B,D) GABA-evoked response. Treatment of primary cultures of sympathetic neurons from E11 chicks with recombinant nARIA for two days increases the magnitude of macroscopic currents activated by acetylcholine and appears to decrease the currents gated by GABA.

Figures 14A-14B: nARIA enhances expression of the nAChR subunit genes α3, α5, α7 and ß4 and increases the magnitude of $I_{P (ACh)}$.

(A) Assay of nAChR subunit gene expression in E9 neurons maintained *in vitro* for 3 days and then treated for 24 hrs with 10 μl of recombinant nARIA (left) of ARIA (right) by quantitative RT-PCR. (B) Assay of $I_{P(ACh)}$ in E9 neurons

maintained *in vitro* for 3 days and then treated for 48 hrs with 10 μl of recombinant nARIA or ARIA or antisense construct of each (control). Macroscopic currents evoked by 500μM ACh.   Peak current ($I_{p_{\prime}Ac_{\hbar}}$) analyzed with non

5   parametric tests appropriate for non-normally distributed values. Box-plots reveal the mid 50% of the data, whiskers delineate the 90% distribution. * indicates outliers. The nARIA induced increase and ARIA induced decrease in ($I_{p_{(Ac_{\hbar}}}$) peak current are significant vs antisense controls at

10   p<0.01, respectively.

Figure 15: Comparison of the affinity of nARIA vs ARIA for heparin sulfate proteoglycan.

MCF7 breast tumor cells were treated with conditioned media

15   from either ARIA or nARIA transiently transfected COS1 cells.   Some of the media was prebound with heparin attached to glass beads.   Prior to treatment, the beads were pelleted by centrifugation to remove any heparin associated proteins. The supernatant was used to treat the MCF7 cells and

20   tyrosine phosphorylation of the ARIA/nARIA receptor was analyzed.

Figure 16: The anti-nARIA antibody is specific for recombinant nARIA

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Figure 17: Anti-nARIA antibodies reveal targeting of nARIA to axon terminals at CNS and PNS synapses

Figure 18: nARIA induction of nAChR expression is more

30   **potent than the Ig-containing isoforms.**

Figure 19: Deletion of nARIA with isoform-specific antisense oligonucleotides reveals that nARIA is both necessary ans sufficient for the regulation of nAChR expression by

presynaptic input.

~~Figures shown in sheets number 26/54 to 54/54 correspond to~~
~~Figures referred to in pages 65~~

(new)

Figure 20: Disruption of CRD-NRG-1 gene products.

(A) Schematic diagram of â-form *neuregulin-1* gene products (.â-forms differ from á-forms in the EGF-like domain). Splice variants of the NRG-1 gene and the various nomenclatures used in the literature are shown. a,b,c refer to three variants of the cytoplasmic domain.

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Ext, extracellular, Tm, transmembrane, CYTO, cytoplasm.

(B) Schematic diagram of the wild-type CRD-containing allele, the targeting vector, and the mutated allele. The barbell indicates the site of the nonsense mutation/Xba I site. Arrow and circled X schematically indicate that the nonsense mutation creates a translation stop codon. Note that the neo cassette is inserted in the antisense orientation into the intron 3' to the CRD-exon.

(C) Southern blot analysis of genomic DNA. Left. EcoRI/Eco RV double digest, with probe A; Right, Xba I digest, with probe B.

(D) Southern Blot analysis of cDNA to examine neonatal neuregulin-1 transcripts. Top. Schematic representation of CRD and Ig containing neuregulin-1 cDNAs. Arrowheads are per primers; solid bands are Ig and TM oligo probes used for Southerns; hatched area is CRD-specific otigo probe. Note the presence of CRD-containing, Ig-containing, and EGF-TM-cytoplasmic containing transcripts in CRD-NRG-1 $^{(+/+)}$ and CRD-NRG-1 $^{(-/-)}$ mice and that CRD transcripts are digested with Xba I only in CRD-NRG-1 $^{(-/-)}$ mice.

(E) a Newborn CRD-NRG-1 $^{(-/-)}$ mice lack spontaneous movement and are cyanotic. b,b' Whole-mount in situ hybridization with CRD-specific probe of Ell CRD-NRG-1 $^{(+/-)}$ & $^{(-/-)}$ mice. N5g, trigeminal: N10g, vagal ganglia; sc, spinal cord; drg, dorsal root ganglia. c,c' Paraffin-section of PO lung stained with H&E. (c) asterisks indicate expanded alveoli in CRD-NRG-T $^{(+/-)}$ mice, (c') carets point to collapsed airspaces in CRD-NRG-1 $^{(-/-)}$ mice.

Figure 21: Defective Neuromuscular Synaptogenesis in CRD-NRG-1 (-/-) mice.

(A) **a,a'** Immunofluorescence labeling (100X) of PO diaphragm with anti-neurofilament antibodies (red) or with **b,b'** anti-neurofilament antibodies (red) & á-bungarotoxin (áBgTx) (green). (**a'**) arrows point to remnant neurofilament(+) staining in mutants, carets (**b**) and arrow(**b'**) point to colocalization of neurofilament and áBgTx staining. **c,c'** Acetylcholinesterase staining (AChE) (400X) of PO diaphragm, (**c**) carets point to strong, aligned AChE staining in controls, (**c^1**) arrows point to weak, scattered AChE staining in mutant. **d,d'** H&E staining of fresh-frozen PO diaphragm muscle, (**d**) carets indicate eccentric, peripheral nuclei, (**d'**) arrows point to central nuclei. (B) **f,f** Immunofluorescence with anti-neurofilament antibodies (red); **e,e',g,g'** anti-neurofilament and anti-synaptophysin antibodies (red) & áBgTx (green); **h,h'** anti-neurofilament antibodies (green) & anti-S100â antibody (red). (**e,e'**) E18.5 intercostal muscle (400X), (**e**) asterisks point out overlap of neurofilament and áBgTx, (**e'**) arrows indicate examples where staining does not overlap. **f,f** E14.5 diaphragm (100X). (**f**) asterisks indicate normal branching pattern of phrenic nerve and termination of intradiaphragmatic branches in central region of muscle, (**f**) arrows highlight defasciculated axons with irregular branching patterns, repeated neurite crossings, and aberrant extension to the most lateral edges of the diaphragm. **g,g'** E14.5 diaphragm (400X). (**g**) asterisks indicate the organized endplate zone where nerve terminals overlie

clusters of áBgTx-labeled AChRs. (g^j) arrows indicate examples where synaptophysin & áBgTx do not overlap. Note disorganized endplate zone with nerve terminals approaching AChRs from many directions and some nerve terminals passing over áBgTx staining **h.h'** E14.5 diaphragm (400X). arrows in **(h)** indicate S100â(+) cells along peripheral nerves absent in **(h')**.

Figure 22: Abnormal projections and subsequent degeneration of peripheral nerves in CRD-NRG-V $^{(-/-)}$ **mice.** **(A)** **a,a'** *In situ* hybridization with vesicular ACh transporter (VAT)-specific probe of 10 μm sections through cervical enlargement of E18.5 spinal cord (200X). **b** Number of motor neurons present in spinal cord levels C1-C6 in control and mutant embryos (n=3 for each age and genotype). **c,c'** Whole-mount immunostaining of E12.5 limb with â-Tubulin III antibody (70X). **(c)** black and yellow asterisks show splayed nerve terminations of spinal nerves and cutaneous sensory projections in controls, respectively. **(c')** black and yellow arrows indicate bulbous, compact terminations of spinal nerves and cutaneous sensory projections in CRD-NRG-V $^{(-/-)}$ mice, respectively. **(B)** **d,d'** *In situ* hybridization with erbBS-specific probe of 10 μm sections through cervical enlargement of E18.5 spinal cord (200X). **d** asterisks indicate sensory root lined with erbB3(+) cells, **d'** arrows point to the sensory root that is devoid of erbB3(+)cells. **e,e',f,f** Whole-mount immunostaining of E12.5 embryos with â-Tubulin III antibody (70X); Imb, limb. **(e,e')** asterisks and arrows indicate the cervical sensory cutaneous projections. **(f,f)** asterisks and arrows

point to trunk lateral cutaneous sensory branches. **g,g**[j] Immunofluorescence with anti-VAT antibodies of E18.5 hindlimb muscle (green) (200X). Asterisks **(g)** & arrows **(g')** arrows point to nerve terminals. **h,h'** Whole-mount *in*

5 *situ* hybridization of E12 embryos with erbBS-specific probe (70X). **(h)** asterisks point to erb3(+) staining along peripheral nerves emanating from caudal spinal cord levels, **(h')** arrows point to peripheral nerves which show decreased erbB3(+)staining. Note that embryo in **(h')** is

10 older than embryo in **(h)**. **i,i'** Immunofluorescence with anti-neurofilament antibodies (green) & anti-S100â antibody (red) (400X). (i)asterisks indicate colocalization of neurofilament & S100â staining in nerve terminations of control, (i') yellow arrows point to

15 colocalization of neurofilament and S100â along preterminal branches; white arrows point to nerve terminations devoid of S100â staining.

Figure 23: Hindbrain Deficits and Loss of Schwann ceil

20 **precursors in CRD-NRG-1**[(-/-)] **mice.**

(A) a-b *In situ* hybridization of 10 μm transverse serial sections of trigeminal ganglion of E14.5 embryos, **(a)** CRD-NRG-1 -specific probe (100X). **(b)** Pan-NRG-1 -specific probe (100X). c-e *In situ* hybridization of 10 μm coronal

25 serial sections of vibrissa follicles of E18.5 embryos, **(c)** CRD-specific probe (200X); **(d)** erbBS-specific probe (200X); **(e)**erbB4-specific probe (200X). f,g *In situ* hybridization of 10 μm coronal sections of E18.5 embryos with CRD-NRG-1-specific probe, **(f)** trigeminal motor

30 nucleus (200X) **(g)** facial motor nucleus (200X). **(B)** h, h'

Whole-mount immunostaining of E11 embryos (70X)with anti-p-tubulin III antibody, **(h')** Arrows indicate the onset of abnormal branching of cranial nerve projections $N5n_{man}$& N7n. N5g, trigeminal; N7g/N8g, vestibulo-cochlear; N9g, petrosal; N10g, nodose ganglia; op, opthalmic, max, maxillary, man, mandibular branches of N5n, trigeminal nerve; N7n, facial; N10n, vagus; N11n, spinal accessory; N12n, hypoglossal nerve; C1,C2 cervical dorsal root ganglion and cervical spinal cord projections. **i,i'** *In situ* hybridization with erbBS-specific probe of trigeminal ganglion of E14.5 embryos, **(i)** asterisks indicate peripheral projections lined with erbB3(+) cells. T-bar indicates the width of nerve (100X). **(i')** arrows point to the peripheral nerve projection in mutants (100X). **(j,j')** Whole-mount *in situ* hybridization with erbBS- specific probe of E12 embryos (70X). **(j)** black and green arrows point to erbBS-positive cells along N11n and its convergence with N10n and along peripheral nerve projections of cervical dorsal root ganglia, respectively. White arrows point to erbB3(+)positive cells in developing muscle, **(j')** black and green arrows point to the dramatic loss of erbB3(+)-staining along N11n, the coalescence of N10n, and along the projections of cervical dorsal root ganglia, respectively. White arrowhead points to the remaining erbBS staining in developing muscle in CRD-NRG-[(-/-)] mice. **k,k',l,l'** *In situ* hybridization of E18.5 coronal sections through hindbrain with a vesicular ACh transporter (VAT)-specific probe. Note the reduction in size of facial motor nucleus (50X) **(k vs k')** and of trigeminal motor

nucleus (200X) **(I vs I')** and abnormal cell migration (arrows, k') in CRD-NRG-1 $^{(-/-)}$ mice. Aq, aqueduct of IVth ventricle, m, midline, LOT, lateral dorsal tegmenal nucleus; PBr$_t$ parabrachial nucleus; N6n, abducens nucleus;

5 N7n, facial nucleus. Dotted line in **k,k'** indicates the midline, m.

Detailed Description of the Invention

This invention provides an isolated nucleic acid molecule
encoding nARIA. This nucleic acid molecule may encode
5   human nARIA (hnARIA), wherein the nucleic acid comprises the
sequence shown from base 93 to base 758 of Figure 3. This
invention also provides for an isolated nucleic acid
molecule encoding nARIA, wherein the nucleic acid molecule
encodes chicken nARIA (cnARIA) which comprises the sequence
    (SEQ ID NO:1)
10   shown from base 608 to base 1234 of Figure 1. The nucleic
acid molecule may be DNA, cDNA or RNA. The isolated nucleic
acid molecule encoding nARIA includes nucleic acids encoding
functionally equivalent variants or mutants of nARIA
including nucleic acid molecules which, due to the
15   degeneracy of the genetic code, code for nARIA polypeptide,
such as the polypeptides shown in Figures 2 and 4 (SEQ ID NO:4)
    (SEQ ID NO:2)

The isolated nucleic acid molecule encoding nARIA includes
nucleic acids encoding biologically active variants of
20   nARIA. This includes nucleic acid molecules which are
capable of specifically hybridizing with an nARIA sequence.
Biologically active variants may include nucleic acid
variants which have at least 75% amino acid sequence
identity with an nARIA sequence, more preferably at least
25   80%, even more preferably at least 90% and most preferably
at least 95%. Identity or homology with respect to an nARIA
sequence is defined herein as the percentage of amino acid
residues in the candidate sequence that are identical with
    (SEQ ID NO:2) (SEQ ID NO:4)
nARIA residues in Figures 2 and 4, after aligning the
30   sequences and introducing gaps, if necessary, to achieve the
maximum percent homology, and not considering any
conservative substitutions to be identical residues. None
of N-terminal, C-terminal or internal extensions, deletions,
or insertions into nARIA sequence shall be construed as
35   affecting homology. The isolated nucleic acid molecule

encoding nARIA also includes any splice variants having nARIA biological activity as defined hereinafter.

As used herein, the purified nARIA polypeptide includes
5   biologically active nARIA polypeptides which include each expressed or processed nARIA sequence, fragments thereof having a unique consecutive sequence of at least 5, 10, 15, 20, 25, 30 or 40 amino acid residues as shown in the underlined regions in Figures 2 and 4. _(SEQ ID NO:2)_ _(SEQ ID NO:4)_ Biologically active
10 amino acid variants of nARIA include a polypeptide wherein an amino acid residue has been inserted N- or C- terminal to, or within the nARIA sequence. Amino acid sequence variants include nARIA wherein an amino acid residue has been replaced by another residue, nARIA polypeptides
15 including those containing predetermined mutations by, e.g. site-directed or PCR mutagenesis. nARIA includes nARIA from such species as rabbit, rat, porcine, non-human primate, Drosophila, equine, murine, opine, human and chicken and alleles or other naturally occurring variants of the
20 foregoing; derivatives of nARIA wherein it has been covalently modified by substitution, chemical, enzymatic or other appropriate means with a moiety such as an enzyme or radioisotope. nARIA may be labeled with a detectable moiety including a fluorescent label, a biotin, a digoxigenin, a
25 radioactive atom, a paramagnetic ion, and a chemiluminescent label. This invention also provides for glycosylation variants of nARIA (as in the insertion of a glycosylation site or deletion of any glycosylation site by deletion, insertion or substitution of an appropriate residue); and
30 soluble forms of nARIA, such as nARIA which lacks a functional transmembrane domain.

As used herein, the purified nARIA polypeptide includes amino acid variants of nARIA which are prepared by
35 introducing appropriate nucleotide changes into nARIA

nucleic acid or by *in vitro* synthesis of the desired nARIA polypeptide. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence shown for human nARIA sequence. Any

5   combination of deletions, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational modifications of nARIA, such as changes in the glycosylation

10   sites, altering the membrane anchoring characteristics, altering the location of nARIA by inserting, deleting or otherwise affecting the transmembrane sequence of native nARIA or modifying its susceptibility to proteolytic cleavage.

15

This invention also provides for fusion proteins which contain nARIA polypeptide linked to an unrelated protein domain(s). The fusion proteins may be created by the insertion of amino- and/or carboxyl-terminal fusions ranging

20   in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e. insertions within the nARIA sequence) may range generally from about 1 to 10 residues,

25   or preferably 1 to 5, and most preferably 1 to 3. Examples of terminal insertions include nARIA with an N-terminal methionyl residue (an artifact of the direct expression of nARIA in bacterial recombinant cell culture), and fusion of a heterologous N-terminal signal sequence to the N-terminus

30   of nARIA to facilitate the secretion of mature nARIA from recombinant host cells. Such signal sequences generally will be obtained from, and thus be homologous to, the intended host cell species. Suitable sequences include STII or 1 pp for *E. coli*, alpha factor for yeast, and viral

signals such as herpes gD for mammalian cells.

Other insertional variants of nARIA may include the fusion
of the N- or C-terminus to an immunogenic polypeptide, e.g.,
5   bacterial polypeptides such as beta-lactamase or an enzyme
encoded by the *E. coli trp* locus, or yeast protein, bovine
serum albumin, or chemotactic polypeptides.

As used herein, the purified nARIA polypeptide includes
10 amino acid substitution variants. These variants have at
least one amino acid residue in the nARIA molecule removed
and a different residue inserted in its place. The sites of
greatest interest for substitutional mutagenesis include a
site(s) identified as an active site(s) of nARIA, and sites
15 where the amino acids found in nARIA ligands from various
species are substantially different in terms of side-chain
bulk, charge, and/or hydrophobicity.

The amino terminus region of the cytoplasmic region of the
20 nARIA may be fused to the carboxy terminus of heterologous
transmembrane domains and receptors, to form a fusion
polypeptide useful for intracellular signalling of a ligand
binding to the heterologous receptor.

25 Other sites of interest are those in which particular
residues of the purified nARIA polypeptide obtained from
various species are identical. These positions may be
important for the biological activity of nARIA. These
sites, especially those falling within a sequence of at
30 least three other identically conserved sites, are
substituted in a relatively conservative manner. Such
conservative substitutions may be known as "preferred
substitutions" and may include: valine substituted for
alanine; lysine for arginine; glutamine for asparagine;

glutamate for aspartate; serine for cysteine; asparagine for glutamine, aspartate for glutamate; proline for glycine; arginine for histidine; leucine for isoleucine; arginine for lysine, leucine for methionine, leucine for phenylalanine; glycine for proline, threonine for serine; serine for threonine; tyrosine for tryptophan; phenylalanine for tyrosine and leucine for valine.

Substantial modifications in function or immunological identity of the purified nARIA polypeptide are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side-chain. Naturally occurring residues are divided into groups based on common side chain properties: hydrophobic; neutral hydrophilic; acidic; basic; residues that influence chain orientation and aromatic. Non-conservative substitutions will entail exchanging a member of one of these classes for another. Such substituted residues may be introduced into regions of nARIA that are homologous with other receptor ligands, or, more preferably, into the non-homologous regions of the molecule.

This invention provides for the creation of a combinatorial library of potential nARIA homologs which can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes can then be ligated into an appropriate vector for expression. The synthesis of degenerate oligonucleotides is well known in the art (see Sambrook, et al., 1989; U.S. Patents Nos. 5,223,409; 5,198,346 and 5,096,815. The purpose of making such a library is to provide in one mixture, all of the

sequences encoding the desired set of potential nARIA sequences. This mixture could then be used for seletion of particular affinities, binding properties and separate functionalities.

5

This invention also provides for a replicable vector which contains nARIA sequence and a host cell containing this vector. This expression vector may be a prokaryotic expression vector, a eukaryotic expression vector, a

10 mammalian expression vector, a yeast expression vector, a baculovirus expression vector or an insect expression vector. Examples of these vectors include PKK233-2, pEUK-Cl, pREP4, pBlueBacHisA, pYES2, PSE280 or pEBVHis. Methods for the utilization of these replicable vectors may be found

15 in Sambrook, et al., 1989 or in Kriegler 1990. The host cell may be a eukaryotic cell, a somatic cell, a germ cell, a neuronal cell, a myocyte, a mammary carcinoma cell, a lung cell, a prokaryotic cell, a virus packaging cell, or a stem cell.

20

This invention also provides for a purified polypeptide or an isolated polypeptide encoding nARIA. This polypeptide may encode human nARIA protein which includes the sequence (SEQ ID NO: 4) shown from amino acid 31 to amino acid 252 of Figure 4A.

25 This polypeptide may also encode chicken nARIA protein which includes the sequence shown from amino acid 203 to amino (SEQ ID NO: 2) acid 421 of Figure 2. This invention provides for the genomic sequence of human nARIA and chicken nARIA. This invention provides for functionally equivalent variants or

30 mutants of nARIA including polypeptides which contain replacement amino acids which do not affect the functionality of the polypeptide. These variants may be prepared using *in vitro* mutagenesis techniques, polymerase chain reaction mutagenesis, or site-directed mutagenesis.

The invention also provides for nARIA derivatives *in vitro* which are immobilized on a support for purposes of diagnoses, purification of nARIA binding factors or affinity purification of nARIA antibodies.

5

According to the present invention, nARIA or <u>n</u>euronal <u>A</u>cetylcholine <u>R</u>eceptor <u>I</u>nducing <u>A</u>ctivity possesses certain biological activities. As used herein, nARIA has the ability to increase both nAChR currents and nAChR subunit

10   gene expression in LSG neurons. The sequence of nARIA is unique throughout the N-terminal portion of the sequence, lacking the Ig-like domain typically upstream of the EGF-like domain in ARIA. nARIA retains the juxtamembrane EGF-like domain, shown to be sufficient for receptor binding and

15   activation of receptor tyrosine kinase activity. Expression of nARIA by Northern blot analysis begins at E4 and is maximal at E8, while expression of ARIA begins at E6 and peaks at E8 in the chick embryo. In cultured E11 chick sympathetic neurons treated with cultured media from COS

20   cells transiently transfected with either ARIA or nARIA, an assay of both acetylcholine (ACh) gated currents and subunit mRNA levels was performed. These assays demonstrated differential regulation of nAChRs by nARIA vs ARIA. Specifically, nARIA significantly increased the maximal

25   responses to 500 mM ACh whereas ARIA significantly decreased the maximal responses compared to cultures treated with recombinant protein from the antisense construct. Measurement of nAChR subunit mRNA levels in E9 sympathetic neurons treated with nARIA or ARIA with quantitative RT-PCR

30   revealed different profiles of subunit gene regulation. A 24 hour treatment with nARIA mimicked the effects of innervation, up-regulating α3, α5, α7 and ß4 levels, whereas ARIA downregulated ß4 and α3. nARIA, therefore, may participate in the increase in nAChR subunit transcription

induced by innervation of embryonic sympathetic neurons *in vivo* during sympathetic neuronal development.

nARIA, as discussed herein, not only has a unique N-terminal
5   region but also displays biological activity distinct from
ARIA.   Tables I, II and III in the Experimental Details
section herein describe these distinctions. nARIA, unlike
ARIA, is specifically expressed only in nervous tissue,
whereas ARIA can be expressed in other tissues.   nARIA
10   expression is higher in the spinal cord and cerebellum than
in the forebrain and optic tectum.   nARIA expression is
first detected at stage E4 in the spinal cord, and
expression is first detected in the cerebellum at stage E8.
In E11 sympathetic neurons, nARIA specifically has an effect
15   on ligand gated channels: in response to acetylcholine,
nARIA specifically increases the number of functional
acetylcholine receptors as indicated by an increase in
response to maximal concentrations of acetylcholine. nARIA
has little effect on the number of GABA activated channels
20   as indicated by the response to maximal concentrations of
GABA.   In contrast, at E11, ARIA has little effect on
acetylcholine evoked resonses and may upregulate GABA evoked
responses.   The effects of nARIA and ARIA on the
acetylcholine evoked responses indicates that nARIA has been
25   quantitated at E9 and is about 15 times more potent than
ARIA.

nARIA increases the transcription of the $\alpha 3$ subunit of nAChR
in sympathetic neurons.   nARIA also increases $\alpha 5$, $\alpha 3$, $\alpha 7$ and
30   ß4 subunit gene expression of nAChR.

This invention provides for a method of inducing the
expression of a specific nicotinic acetylcholine receptor
subunit isoform.   AChRs at mature mammalian neuromuscular

junctions are pentameric protein complexes composed of four subunits in the ratio of $\alpha_2\beta\epsilon\delta$ (Mishina et al 1986). Most, if not all, of embryonic AChRs contain a different subunit, termed "τ" in place of the ε subunit. When mixtures of

5   α,β,δ and τ subunit mRNAs are injected into *Xenopus* oocytes, the expressed channels have the properties of embryonic receptors. It is likely that this change in subunit composition is due to a change in gene expression and accounts for the switch in properties of ACh-activated

10   channels from slow channels to fast channels. This invention provides for the application of nARIA alone or in combination with another agent to neural cells to induce the expression of subunit isoforms.

15   This invention further provides for nARIA antagonists which are capable of reducing the biological activity of nARIA. This antagonist may be proteinaceous such as an antibody specific for nARIA as described herein, a nucleic acid such as an antisense molecule to the nARIA mRNA as described

20   herein, an enzymatic activity such as a ribozyme directed to nARIA mRNA as described herein or a protease specific for the nARIA polypeptide. The antagonist may also be an agent which is capable of binding the nARIA receptor with higher affinity than nARIA, thus competing away the effects of

25   nARIA binding.

The subject invention also provides for nARIA agonist(s) which would be capable of enhancing the biological activity of nARIA. Such agonists may include other neurotrophic

30   factors such as ciliary neurotrophic factor (see U.S. Patent No. 4,997,929); nerve growth factor (see U.S. Patent No. 5,169,762); neurotrophic factor 4/5 (see PCT International Publication No. WO 92/05254); brain-derived neurotrophic factor (see U.S. Patent No. 5,180,820); glial-derived

neurotrophic factor (see PCT International Publication No. WO 93/06116) or any other neurotrophic factor (see European application EP 0 386 752 A1). The disclosures of these publications in their entireties are hereby incorporated by
5   reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

10   This invention also provides for an antibody immunoreactive with an epitope comprising a unique sequence shown in either Figure 2 (SEQ ID NO:2) from amino acid 203 to amino acid 421 or in Figure 4 (SEQ ID NO:4) from amino acid 31 to amino acid 252.

15   A further embodiment of the invention is a monoclonal antibody which is specific for nARIA. In contrast to conventional antibody (polyclonal) preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody
20   is directed against a single determinant on the antigen. Monoclonal antibodies are useful to improve the selectivity and specificity of diagnostic and analytical assay methods using antigen-antibody binding. Also, they may be used to remove nARIA from the serum. A second advantage of
25   monoclonal antibodies is that they can be synthesized by hybridoma cells in culture, uncontaminated by other immunoglobins. Monoclonal antibodies may be prepared from supernatants of cultured hybridoma cells or from ascites induced by intraperitoneal inoculation of hybridoma cells
30   into mice. The hybridoma technique described originally by Köhler and Milstein, 1976, has been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens.

35   Another embodiment of this invention is a ribozyme which is

capable of cleaving nARIA mRNA. See Cech, et al., U.S. Patent No. 4,987,071; Altman et al., U.S. Patent No. 5, 168,053; Haseloff et al, U.S. Patent No. 5,254,678 published European application No. Hampel et al., EP
5   360,257.

This invention provides for a nucleic acid comprising a unique nARIA sequence in a 3' to 5' orientation, antisense to at least a portion of a gene encoding naturally occurring
10   nARIA. This antisense nucleic acid molecule may be labeled with a detectable moiety selected from the group consisting of a fluorescent label, a biotin, a digoxigenin, a radioactive atom, a paramagnetic ion, and a chemiluminescent label. See Inoue et al. U.S. Patent Nos. 5,208,149 and
15   5,190,931 and Schewmaker, U.S. Patent No. 5,107,065.

Labeling of a circular oligonucleotide (such as a replicable vector as described herein) can be done by incorporating nucleotides linked to a "reporter molecule" into the subject
20   circular oligonucleotides. A "reporter molecule", as defined herein, is a molecule or atom which, by its chemical nature, provides an identifiable signal allowing detection of the circular oligonucleotide. Detection can be either qualitative or quantitative. The present invention
25   contemplates using any commonly used reporter molecule including radionucleotides, enzymes, biotins, psoralens, fluorophores, chelated heavy metals, and luciferin. The most commonly used reporter molecules are either enzymes, fluorophores, or radionucleotides linked to the nucleotides
30   which are used in circular oligonucleotide synthesis. Commonly used enzymes include horseradish peroxidase, alkaline phosphatase, glucose oxidase and α-galactosidase, among others. The substrates to be used with the specific enzymes are generally chosen because a detectably colored
35   product is formed by the enzyme acting upon the substrate.

For example, p-nitrophenyl phosphate is suitable for use with alkaline phosphatase conjugates; for horseradish peroxidase, 1.2-phenylenediamine, 5-aminosalicylic acid or toluidine are commonly used. The probes so generated have

5 utility in the detection of a specific nARIA DNA or RNA target in, for example, Southern analysis, Northern analysis, *in situ* hybridization to tissue sections or chromosomal squashes and other analytical and diagnostic procedures. The methods of using such hybridization probes

10 are well known and some examples of such methodology are provided by Sambrook et al, 1989. This invention also provides a method of amplifying a nucleic acid sample comprising priming a nucleic acid polymerase chain reaction with nucleic acid (DNA or RNA) encoding (or complementary

15 to) an nARIA.

Another embodiment of this invention is the normal expression or overexpression of nARIA *ex vivo* in human neuronal cells, stem cells or undifferentiated nerve cells

20 and muscle cells. These cells may be utilized for gene therapy in patients (See Anderson et al U.S. Patent No. 5,399,346).

This invention further provides for a transgenic nonhuman

25 mammal whose germ or somatic cells contain a nucleic acid molecule which encodes nARIA polypeptide or biologically active variants thereof, introduced into the mammal, or an ancestor thereof, at an embryonic stage. This invention provides for a transgenic nonhuman mammal whose cells may be

30 transfected with a suitable vector with an appropriate sequence designed to reduce expression levels of nARIA polypeptide below the expression levels of that of a native mammal. The transgenic nonhuman mammal may be transfected with a suitable vector which contains an appropriate piece

of genomic clone designed for homologous recombination. Alternatively, the transgenic nonhuman mammal may be transfected with a suitable vector which encodes an appropriate ribozyme or antisense molecule. See for example, Leder and Stewart, U.S. Patent No. 4,736,866 for methods for the production of a transgenic mouse.

Biologically functional variants of nARIA are nucleic acid molecules that, due to the degeneracy of the genetic code, code on expression for nARIA polypeptide. The foregoing variant DNA sequences may be translated into variant nARIA polypeptides which display the biological activity of an nARIA polypeptide. These variant nucleic acid molecules may also be expressed in this transgenic mammal. Active variants should hybridize to the wild-type nARIA nucleic acid sequence under highly stringent or moderately stringent conditions (Sambrook et al, 1989).

One embodiment of this invention is a method for inducing the formation of a synaptic junction between a neuron and a target cell, which includes treating the target cell with nARIA polypeptide or nARIA nucleic acid molecule encoding nARIA or a biologically active variant thereof, in an amount sufficient to induce the formation of a synaptic junction. A "sufficient amount" as used herein refers to that amount which provides a therapeutic effect for a given condition and administration regimen. The target cell may be a somatic cell such as a myocyte, a neuronal cell, a glandular cell or any postsynaptic cell. This method provides for the induction of the formation of a synaptic junction in an individual having a neurological disorder involving abnormal synaptic connections. Isolated nARIA may be used as a growth factor for *in vitro* cell culture or *in vivo* to promote the growth of cells.

nARIA, nARIA agonists or nARIA antagonists may be used to treat any disease where levels of nARIA metabolism are changed and therefore ion channel levels or activities are not normal as in some neurological disorders. Such

5 disorders would include any disease with an abnormal production of nARIA. Neurological disorders that affect the central nervous system, memory or cognitive functions may also be treated with nARIA. Such disorders may be the result of the normal aging process or the result of damage

10 to the nervous system by trauma, surgery, ischemia, infection or metabolic disease. Such disorders may also include Alzheimer's Disease, Turret's Syndrome, and Parkinson's Disease. These diseases have been shown to respond to nicotine treatment.

15

The neurological disorder may be a neuromuscular disorder. Examples of neuromuscular disorders which may be treatable with nARIA include Alzheimer's disease, myasthenia gravis, Huntington's disease, Pick's disease, Parkinson's disease,

20 and Turret's Syndrome. Also included are neurogenic and myopathic diseases including chronic atrophies such as amyotrophic lateral sclerosis, Guillain-Barre syndrome, progressive bulbar palsies, spinal muscular atrophies and chronic peripheral neuropathy. Autonomic disorders of the

25 peripheral nervous system may also be included in this treatment which include disorders that affect the innervation of muscle or endocrine tissue such as tachycardia, atrial cardiac arrhythmias and hypertension. These disorders are thought to be associated with an

30 abnormally low level of muscarinic AChRs in the striated muscle.

This invention provides for a method of altering neuro-receptor expression. In this method, nARIA is administered

35 to a subject which may result in a change in the expression

of neuro-receptors. The method of administration of nARIA is described more fully hereinafter.

This invention provides for the production of functional
5   mammalian nARIA protein in a prokaryotic expression system, a mammalian expression system, a baculovirus expression system, an insect expression system or a yeast expression system. This production may provide for the post-translational modifications which exist in the naturally
10   occurring nARIA protein. For protocols describing bacterial expression of mammalian proteins, see Sambrook et al, 1989.

Another embodiment of this invention is a method for inducing neuronal regeneration which comprises contacting a
15   target cell with a composition of nARIA and a pharmaceutically acceptable carrier to induce the formation of a synaptic junction between a neuron and a target cell. The target cell may be a neuronal cell, an endocrine cell, a muscle cell or any cell capable of forming a neuro-
20   muscular junction. nARIA may be used to facilitate incorporation of implants into nervous tissue or to promote nerve regeneration following damage by trauma, infarction, infection or postoperatively.

25   This invention provides for a combination therapy of nARIA with another neurotropic factor or cytokine or growth factor or with other agents known for use in the treatment of malignancies. Such factors may include transforming growth factor beta (TGF-ß), ciliary neurotropic factor (CNTF),
30   brain derived neurotropic factor (BDNF), NT-4, NT-5, NT-4/5, nerve growth factor (NGF), activins, agrin, cell differentiation factor (CDF), glial growth factor (GGF), and neu differentiation factor (NDF), ARIA, and heregulins. nARIA may be administered in combination with agrin for
35   effects on the neuromuscular junction. For therapy directed

toward the autonomic/enteric nervous system, TGF-ß and nARIA is the preferred combination. For therapy directed to the central nervous system, nARIA and CDF is the preferred combination.

5

When administered parenterally, proteins are often cleared rapidly from the circulation and may therefore elicit relatively short-lived pharmacological activity. Consequently, frequent injections of relatively large doses

10 of bioactive proteins may by required to sustain therapeutic efficacy. Proteins modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol,

15 polyvinylpyrrolidone or polyproline are known to exhibit substantially longer half-lives in blood following intravenous injection than do the corresponding unmodified proteins (Abuchowski et al., 1981; Newmark et al., 1982; and Katre et al., 1987). Such modifications may also increase

20 the protein's solubility in aqueous solution, eliminate aggregation, enhance the physical and chemical stability of the protein, and greatly reduce the immunogenicity and antigenicity of the protein. As a result, the desired *in vivo* biological activity may be achieved by the

25 administration of such polymer-protein adducts less frequently or in lower doses than with the unmodified protein. nARIA compositions may be administered parenterally by injection or directly into the cerebral spinal fluid by continuous infusion from an implanted pump.

30 nARIA may also be administered with one or more agents capable of promoting penetration of nARIA across the blood-brain barrier.

Attachment of polyethylene glycol (PEG) to proteins is

particularly useful because PEG has very low toxicity in mammals (Carpenter et al., 1971). For example, a PEG adduct of adenosine deaminase was approved in the United States for use in humans for the treatment of severe combined

5   immunodeficiency syndrome. A second advantage afforded by the conjugation of PEG is that of effectively reducing the immunogenicity and antigenicity of heterologous proteins. For example, a PEG adduct of a human protein might be useful for the treatment of disease in other mammalian species

10   without the risk of triggering a severe immune response. nARIA or cells that produce nARIA may be delivered in a microencapsulation devise so as to reduce or prevent an host immune response against the nARIA producing cells. nARIA may also be delivered microencapsulated in a membrane, such

15   as a liposome.

Polymers such as PEG may be conveniently attached to one or more reactive amino acid residues in a protein such as the alpha-amino group of the aminoterminal amino acid, the

20   epsilon amino groups of lysine side chains, the sulfhydryl groups of cysteine side chains, the carboxyl groups of aspartyl and glutamyl side chains, the alpha-carboxyl group of the carboxy-terminal amino acid, tyrosine side chains, or to activated derivatives of glycosyl chains attached to

25   certain asparagine, serine or threonine residues.

Numerous activated forms of PEG suitable for direct reaction with proteins have been described. Useful PEG reagents for reaction with protein amino groups include active esters of

30   carboxylic acid or carbonate derivatives, particularly those in which the leaving groups are N-hydroxysuccinimide, p-nitrophenol, imidazole or 1-hydroxy-2-nitrobenzene-4-sulfonate. PEG derivatives containing maleimido or haloacetyl groups are useful reagents for the modification

35   of protein free sulfhydryl groups. Likewise, PEG reagents

containing amino hydrazine or hydrazide groups are useful for reaction with aldehydes generated by periodate oxidation of carbohydrate groups in proteins.

5   Another embodiment of this invention is a method for determining a prognosis of or diagnosing a neoplastic condition in a subject. In this method, one may obtain a biological sample from the subject, and contact the sample with a reagent capable of binding to an element in the

10   sample, the element being an nARIA nucleic acid molecule or polypeptide encoding nARIA, under conditions such that the reagent binds only if the element is present in the sample. One may then detect the presence of the reagent bound to the element and thereby determine the prognosis of the

15   neoplastic condition of the subject. The reagent may be an oligonucleotide capable of hybridizing with a nucleic acid encoding nARIA polypeptide under standard stringency hybridization conditions. The reagent in this method may be an antibody specific for nARIA polypeptide. The element in

20   the biological sample may be a nucleic acid molecule encoding nARIA or a polypeptide encoding nARIA protein. The biological sample may be cerebrospinal fluid, blood, plasma, ascites fluid, tissue, urine, sputum, amniotic fluid, saliva, lung lavage, or cell extracts. This method may be

25   performed with the reagent is affixed to a solid support. The neoplastic condition may be a mammary neoplasm or a small cell carcinoma of the lung.

A further embodiment of this invention is a method for the

30   treatment of a neoplastic condition of a subject. In this method a pharmaceutically acceptable form of nARIA in a sufficient amount over a sufficient time period is administered to a subject to induce differentiation of neoplastic cells and thus treat the neoplastic condition.

35   The composition may be a form of nARIA such as nARIA

polypeptide or nARIA nucleic acid, combined with a pharmaceutically acceptable carrier. The carrier may be made up of suitable diluents, preservatives, solubilizers, emulsifiers, or adjuvants and may be in an aerosol,
5   intravenous, oral or topical form.

Also provided by the invention are pharmaceutical compositions comprising therapeutically effective amounts of polypeptide products of the invention together with suitable
10   diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. A "therapeutically effective amount" as used herein refers to that amount which provides a therapeutic effect for a given condition and administration regimen. Such compositions are liquids or
15   lyophilized or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCl., acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile
20   acid salts), solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of
25   polymers such as polyethylene glycol to the protein, complexation with metal ions, or incorporation of the material into or onto particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, hydrogels, etc, or onto liposomes, microemulsions, micelles,
30   unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance of nARIA. The choice of compositions will depend on the physical and chemical

properties of the protein having nARIA activity. For example, a product derived from a membrane-bound form of nARIA may require a formulation containing detergent. Controlled or sustained release compositions include
5   formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., poloxamers or poloxamines) and nARIA coupled to antibodies directed against tissue-specific receptors, ligands or antigens or
10   coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal,
15   oral, injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial or intralesional. nARIA may be part of a pharmaceutical composition with agrin and an acceptable carrier to recapitulate both the induction of expression of AChR and
20   the clustering of the AChR's on the membrane surface.

Polypeptides of the invention may be "labeled" by association with a detectable marker substance (e.g., radiolabeled or biotinylated) to provide reagents useful in
25   detection and quantification of nARIA or its receptor bearing cells in solid tissue and fluid samples such as blood or urine.

Another embodiment of this invention is a method for
30   determining whether a compound is capable of modulating the binding of an nARIA polypeptide to its receptor. In this method, the compound may be incubated under suitable conditions with an appropriate nARIA polypeptide-affinity derivative or receptor-affinity derivative under appropriate
35   conditions such that an affinity complex may form. Then,

one may measure the amount of affinity complex formed so as to determine whether the compound is capable of modulating the binding of the nARIA polypeptide to its receptor. The affinity complex may be an nARIA receptor bound to an affinity derivative or an nARIA polypeptide bound to a derivative. The measurement in this method may comprise binding of an antibody specific for nARIA to the affinity complex to measure the amount of affinity complex formed. The affinity derivative may be sepharose, cellulose, plastic, glass, latex, glass beads, a nylon membrane, a cellulose acetate membrane, an epoxy-activated synthetic copolymer membrane, a nitrocellulose membrane or a streptavidin-coated plastic.

The present invention provides an assay for diagnosing whether a subject has or is predisposed to developing a neoplastic disease which comprises: a) obtaining a biological sample from the subject; b) contacting the sample with an agent that detects the presence of an extracellular domain of nARIA (CRD-neuregulin) or an isoform thereof; c) measuring the amount of agent bound by the sample; d) comparing the amount of agent bound measured in step c) with the the amount of agent bound by a standard normal sample, a higher amount bound by the sample from the subject being indicative of the subject having or being predisposed to developing a neoplastic disease.

The present invention also provides assay for determining whether a subject has a neurodegenerative diseaase which comprises: a) obtaining a biological sample from the subject; b) contacting the sample with an agent that detects the presence of an extracellular domain of nARIA (CRD-neuregulin) or an agent which detects the presence of soluble neuregulin receptor; c) measuring the amount of agent bound by the sample; d) comparing the amount of bound

agent measured in step c) with the the amount of agent bound by a standard normal sample, a higher amount or a lower amount bound by the sample from the subject being indicative of the subject having a neurodegenerative disease.

5

In one embodiment, the agent is an antibody or a fragment thereof. In another embodiment, the sample is cerebrospinal fluid (CSF), blood, plasma, sputum, amniotic fluid, ascites fluid, breast aspirate, saliva, urine, lung lavage, or cell

10 lysate or extract derived from a biopsy. In a further embodiment, the agent is an antibody which binds to an epitope formed by the amino acid sequence shown in Figure 2 (SEQ ID NO: 2) or Figure 4 (SEQ ID NO: 4) In another embodiment, the agent is an antibody which binds to an epitope of the cytoplasmic domain

15 of nARIA. In another embodiment, the agent is an antibody which specifically binds to the amino acid sequence NQDPIAV (Seq ID No. 5). In a further embodiment, the neoplastic disease is breast cancer, prostate cancer, brain cancer or ovarian cancer. In another embodiment, the

20 neurodegenerative disease is Alzheimer's Disease, Parkinson's disease, Turrets Syndrome, amyotrophic lateral sclerosis, Pick's disease, myasthenia gravis, or senility.

The present invention also provides a method for maintaining

25 or sustaining a synaptic connection between a neuron and a target cell comprising contacting the target cell with an nARIA polypeptide or a nucleic acid molecule encoding nARIA or biologically active variant thereof, in an amount sufficient to maintain the synaptic junction.

30

In one embodiment, the target cell is a somatic cell including a myocyte, a neuronal cell, a glandular cell or any postsynaptic cell. In another embodiment, maintenance of the synaptic junction is accomplished in an individual

35 having a neurological disorder involving abnormal synaptic

connections. In another embodiment, the neurological disorder is a neuromuscular disorder or a neurodegenerative disease. In a further embodiment, the nARIA polypeptide is NQDPIAV (Seq ID No. :5) or the A-form of the cytoplamic
5   domain of nARIA (Seq ID No.   ). In a further embodiment, the neurological disorder is Alzheimer's Disease, Parkinson's disease, Turrets Syndrome, amyotrophic lateral sclerosis, Pick's disease, myasthenia gravis, or senility.

10   The present invention also provides a method for inducing neuronal regeneration which comprises contacting a target cell with a composition of nARIA and a pharmaceutically acceptable carrier to induce the formation of a synaptic junction between a neuron and a target cell. In one
15   embodiment, the target cell is a neuronal cell or a muscle cell.

The present invention also provides a method for the treatment of a neoplastic condition of a subject which
20   comprises administering to the subject a pharmaceutically acceptable form of nARIA in a sufficient amount over a sufficient time period to induce differentiation of neoplastic cells and thus treat the neoplastic condition.

25   The present invention also provides a method for determining whether a compound is capable of modulating the binding of an nARIA polypeptide to its receptor, which comprises: (a) incubating the compound under suitable conditions with an appropriate nARIA polypeptide-affinity derivative or
30   receptor-affinity derivative under appropriate conditions such that an affinity complex may form; (b) measuring the amount of affinity complex formed so as to determine whether the compound is capable of modulating the binding of the nARIA polypeptide to its receptor.

35

In one embodiment, the affinity complex comprises an nARIA receptor bound to an affinity derivative. In another embodiment, the affinity complex comprises an nARIA polypeptide bound to an affinity derivative. In another
5 embodiment, the measuring comprises binding of an antibody specific for nARIA to the affinity complex to measure the amount of affinity complex formed. In another embodiment, the affinity derivative comprises sepharose, cellulose, plastic, glass, glass beads, or a streptavidin-coated
10 plastic.

The present invention also provides an assay for detecting neoplastic disease in a subject which comprises: a) obtaining a biological sample from the subject; b)
15 contacting the sample with an agent that specifically binds to an expression product of a neuregulin gene or a neuregulin receptor; c) measuring the amount of agent bound by the sample; d) comparing the amount of agent bound measured in step c) with the the amount of agent bound by a
20 standard normal sample, a higher amount bound by the sample from the subject being indicative of the presence of neoplastic disease in the subject.

In one embodiment, the neuregulin receptor is erbB2, erbB3
25 or erbB4. In another embodiment, the agent specifically binds to an amino acid sequence of neuregulin which directs translocation to the nucleus. In another embodiment, an expression product of a neuregulin gene comprises a neuregulin protein, an extracellular domain of a neuregulin
30 protein, a polypeptide encoded by the amino acid sequence
   (SEQ ID NO: 2)
shown in Figure 2ʌ or 4. In another embodiment, the agent is
                    ʌ
detectably labelled. (SEQ ID NO: 4)

35

This invention is illustrated in the Experimental Detail section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

The present invention provides an assay for determining the amount of her 2 receptor in a bodily fluid from a

10   subject and an assay for other heregulin receptors which comprises a) obtaining a bodily fluid from a subject or a sample from a subject (i.e. a brain punch biopsy, blood, serum, plasma, urine, etc.); b)contacting the sample with cells which are transfected with a reporter

15   construct (the construct being CRD-neuregulin with a cytoplasmic tail whch has a detetable marker attached to it); c)measuring the detectable label in the nucleus of the cells and thereby determining the amount of her 2 receptor in the bodily fluid from the sample.

20

The neuregulin in the bodily fluid binds to a receptor on the surface of the transfected cell and there will be cleavage of the external domain and the cleavage of the internal cytoplasmic domain. The cytoplasmic domain will

25   then be translocated into the nucleus and the detectable able will be available for detection in the nucleus. The detectable label may be green fluorescent protein or VP16.

30   Another assay which is provided for in the present invention is an assay which comprises obtaining a brain

punch from a subject and performing immunohistochemistry on frozen sections or preserved sections of the tissue obtained using a detectably labeled antibody specific for the cytoplasmic domain of neuregulin and determining

5   the percentage of nuclei that are showing label in the tissue sample.

The nuclear targeting domain of neuregulin may be found in Yang et al and begins at amino acid number 230

10 (KTKKQRKK).

This invention is illustrated in the Experimental Detail section which follows. These sections are set forth to aid in an understanding of the invention but are not

15 intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

Experimental Details

Example 1 - Isolation and Sequence Analysis of nARIA

Novel members of the ARIA/NDF/heregulin family that would be expressed in neurons projecting to cholinoceptive neural targets were identified. Cloning efforts yielded 29 positives, including several which encoded variants with an entirely novel N-terminal sequence, distinguished by the absence of the usual Ig-like domains of the heregulin/NDF family. The predominance of the novel, Ig-less clones over the ARIA like clones was striking (11 vs 5 of 29 positives). We named the novel splice variant "nARIA" for neuronal nAChR Inducing Activity. All clones were isolated by two different and separate approaches as described below.

A different library was used in each screening protocol carried out to isolate and clone the nARIA gene. A chick E13 total brain cDNA library was screened with a rat DNA probe generated by PCR amplification. For the PCR amplification, degenerate primers corresponding to nucleotide sequences 523-542 (upper primer) and 1080-1100 (lower primer) of the published rat NDF sequence (Wen et al., 1992) were used to amplify a DNA fragment from a template of adult rat spinal cord cDNA. The upper and lower primers were within the immunoglobulin and transmembrane domains respectively. The amplified fragment was subcloned into the pCRII$_z$ vector (Invitrogen) and was sequenced, revealing an open reading frame. The predicted peptide encoded by this fragment contained the immunoglobulin to transmembrane domains of the heregulin ß1 isoform and is distinct from the published NDF sequence, which is an α-isoform of heregulin. Screening of the cDNA library by random primed labeling of the amplified fragment resulted in

three independent clones, of which only one contained a complete open reading frame. This open reading frame is 2055 nucleotides (Figure 1, (SEQ IO NO: 1) nucleotides 608-2662) and encodes the nARIA transcript. The nucleotide sequences from
5   base pair 1293 downstream to the poly-A tail of the nARIA clone are identical to ARIA a related cloned chicken gene (Falls et al., 1993).

On the protein level, identical sequences encode the portion
10 of the molecule spanning from the EGF-like domain to the C-terminus in ARIA and nARIA. The break in homology occurs at a known splice site and the sequences upstream to the splice junction are unique to nARIA. Analysis of the predicted protein sequence did not produce a motif corresponding to an
15 immunoglobulin domain. Instead, there was a cysteine rich region identified (8 cysteines in 34 amino acids). See Figure 2, (SEQ IO NO: 2)

Example 2 - Spatial and Temporal Expression of nARIA in
20 chick development

A chick E5-E11 spinal cord cDNA library which we prepared was also screened. The probe for screening was generated by RT-PCR amplification from E8 chick spinal cord total RNA
25 using primers corresponding to nucleotide sequences 264-281 (upper primer) and 1294-1313 (lower primer) of the published ARIA sequence (Falls, et al., 1993). The amplified PCR fragment was subcloned into a PGEM3Z vector and sequenced to confirm its identity. Screening of the chick E5-11 spinal
30 cord primary cDNA library by random primed labeling using the PCR fragment as the template resulted in 26 clones. Of these, 11 contained the novel cysteine rich domain (nARIA like clones), 6 clones contained the immunoglobulin-like domain (ARIA like splice forms), while the remaining clones
35 had unidentifiable sequences. The nARIA clones included 8

that were identical to the form obtained by the earlier screening and 3 additional isoforms that differed in the N-terminus, the EGF-like domain, the juxtamembrane linker, and/or the C-terminal region. The extracellular domains of
5 these clones were fully sequenced and the intracellular portions were partially sequenced. Restriction mapping and Southern blotting were also used to confirm the relatedness of these molecules. A human cerebellar cDNA library was screened in the manner described above. This procedure (SEQ ID No. 3)
10 resulted in the isolation of human nARIA (Figures 3 & 4).
(SEQ ID NO: 4)

Multiple tissue Northern blots were screened with probes specific for unique domains of nARIA and were compared with those probed with an ARIA specific probe (Figure 6). In
15 particular, the ARIA probe detected ARIA in skeletal muscle (pectoral muscle) whereas expression of nARIA was found to be restricted to nervous tissue. The expression of nARIA represents a higher percentage of the total message in the cerebellum and spinal cord than in the forebrain or optic
20 tectum. ARIA message is represented at a higher level in the forebrain and optic tectum than in the cerebellum and spinal cord (Figure 7). The developmental expression patterns of nARIA and ARIA in spinal cord as detected by RT-PCR and Northern blot hybridization are different (Figure
25 8A-8B). The mRNA of nARIA is detectable by E3 and robust by E4 whereas initiation of ARIA and expression occurs later (E6-8) (Figure 9A-C).

In situ hybridization studies with probes specific for nARIA
30 and ARIA (containing the cysteine rich domain or Ig-like domain respectively) also demonstrated different patterns of expression (Figure 10A-D). In particular, a positive signal is obtained in the presumptive preganglionic neurons with the nARIA probe but not with the ARIA probe. Therefore, the

pattern of expression of ARIA and nARIA are different.

Example 3 - Functional Analysis of Biological Activity -
nARIA Activates Tyrosine Kinase Linked Receptor(s).

5

Initial experiments to characterize the functional properties of the nARIA protein focused upon the ability to activate protein tyrosine kinases. ARIA has been proposed to act, as other members of the NDF/heregulin family,
10   through an interaction with specific tyrosine kinase-linked receptors (Falls, et al., 1993). This first step in transduction is assayed as tyrosine phosphorylation of a high molecular weight band, thought to represent phosphorylation of the receptor subunit(s). We examined the
15   pattern of tyrosine phosphorylated proteins in extracts of lumbar sympathetic ganglia (LSG) neurons as well as several other cell lines with an anti-TYR-P antibody (4G10; UBI) (Ausubel et al., 1994; Falls, et al., 1993). Both recombinant ARIA and nARIA (from transiently transfected COS
20   cells) induced time and dose dependent phosphorylation of 170-185 kD bands in the MCF7 and MDA-MB-453 cell lines (human carcinomas that overexpress erb-B2 receptor). ARIA appeared more potent than nARIA and somewhat less robust in phosphorylation of a 185 kD band in LSG. Differential
25   effects of nARIA on glial cells and neurons differ from all other heregulin isoforms including ARIA, examined to date. nARIA's unique N-terminal sequence influences the binding of the isoform to the protein tyrosine kinase receptors thereby conferring distinct specificities.

30

Media conditioned by COS1 or HEK293 cells transiently transfected with the nARIA clone (sense configuration) activated tyrosine kinase activity in the breast tumor cell lines MCF7 or MDA-MB-453 above the basal levels of tyrosine
35   kinase activity as determined by using the antisense

configuration of nARIA. See Figure 11A-C. Furthermore, the levels of phosphorylation of the EGFR family members relative to one another was different between ARIA and nARIA. ARIA treatment resulted in a higher level of
5   erB3/HER3 phosphorylation than nARIA. Treatment of acutely dispersed sympathetic neurons from E9 chicks with nARIA conditioned media resulted in increased tyrosine phosphorylation of an approximately 180 kD protein (Figure 12).

10

Another assay to more clearly delineate between the biological activities of nARIA and ARIA involved the comparison of their effects on the expression of ligand-gated channels in primary neurons. These studies assayed
15   the number of functional surface receptors for two transmitters (ACh and GABA) using an electrophysiological assay of transmitter gated macroscopic currents. The rationale for these experiments is based upon the pattern of expression of nARIA and ARIA, and on our previous studies of
20   receptor regulation by spinal cord neurons (Role, 1988; Gardette et al, 1991). Treatment of primary cultures of sympathetic neurons from E11 chicks with recombinant nARIA for two days increased the magnitude of macroscopic currents activated by acetylcholine and appeared to decrease the
25   currents gated by GABA (Figure 13A-D). In contrast, treatment with recombinant ARIA under the same conditions decreased the currents gated by acetylcholine and appeared to enhance GABA-evoked currents. The differential effects of nARIA vs ARIA on ACh gated currents, an index of the
30   number of functional channels on the cell surface, was also reflected in the assays of the levels of expression of ACh receptor subunit encoding mRNA's.

Notably, a 24 hour treatment with recombinant nARIA
35   increased the level of $\alpha 3$ subunit mRNA; in contrast, the

level of α3 subunit mRNA was either slightly decreased or not altered by ARIA. The differential effect of nARIA and ARIA on transcription was not limited to the acetylcholine receptor subunits. Application of the differential display

5   technique to primary cultures of sympathetic neurons treated for 24 hours with nARIA or ARIA suggested that the two growth factors differentially activate or suppress transcription of several distinct cDNAs.

10   The data presented herein suggests that the novel splice variant of the heregulin gene, nARIA may play a role in synaptic development that is unique from that of ARIA or other immunoglobulin-domain-containing splice variants. nARIA may potentially be used therapeutically or

15   diagnostically. Alterations in the level of the production of nARIA may be indicative of a traumatic insult to the nervous system. Since the receptor for this factor is a known oncogene, changes in growth factor levels may be prognostic to some neoplastic conditions. Recombinant nARIA

20   may be useful in cancer treatment regimens or for use in neuronal regeneration as described more fully herein. Other isoforms have been demonstrated to induce differentiation of breast tumor cell lines, promote survival of glial cells and increase the mitogenesis of some cell lines.

25

It has been demonstrated that the biological activity of nARIA is different than that of ARIA and is summarized in Table I. (The bold serves to highlight the differences in biological activity between ARIA and nARIA.)

30

Table I. Biological Activity and Effects of ARIA vs nARIA

Biological Activities	ARIA	nARIA
Expression	Seen in chick nervous tissues and skeletal muscle **Expression is higher in the forebrain and optic tectum than in the spinal cord and cerebellum** **Expression starts at E6 in the spinal cord** **Expression starts at E8 in the cerebellum**	**Seen only in nervous tissue at E13** **Expression is higher in the spinal cord and cerebellum than in the forebrain and optic tectum** **Expression starts at E4 in the spinal cord** **Expression starts at E8 in the cerebellum**
Activation of p185 phosphorylation	Seen in L6 rat myocytes **Seen in PC12 cells (Fischbach, et al)** Seen in sympathetic neurons Seen in ciliary neurons (Fischbach, et al) Seen in breast tumor cell lines MCF7 and MDA-MB-453 Seen in rat O2A cells (Fischbach)	Seen in L6 rat myocytes **Not seen in PC12 cells** Seen in sympathetic neurons Not determined Seen in breast tumor cell lines MCF7 and MDA-MB-453 Not determined

Effects on ligand gated channels	Increases response to acetylcholine in E9 and **decreases response in E11 sympathetic neurons** Increases response to GABA in E9 and **increases response in E11 sympathetic neurons.** Increases response to ACh in muscle cells (Fischbach et al.)	Increases response to acetylcholine in E9 and **increases response in E11 sympathetic neurons** Increases GABA responses in E9 and **decreases responses in E11 sympathetic neurons** Not determined
Effects on transcription of nAChR subunits in rat medial habenula	No effect	Not determined
Effects on transcription of nAChR subunits in sympathetics	**No effect on $\alpha3$** Increases $\alpha5$ and $\alpha7$	**Increases $\alpha3$** Increases $\alpha5$ and $\alpha7$
Effects on sodium channel in muscle	Increases (Fischbach et al.)	Not determined
Effects on PC12 differentiation	Induces very short neurites in 10% of the cells (Fischbach et al.) Reduces rate of replication by half (Fischbach et al.)	Not determined Not determined
Effects on glial cells	Increases the number of oligodendrocytes that develop from O2A precursors (Fischbach et al.)	Not determined

Effects on nAChR subunits in PC12 cells	"Like control" (Fischbach et al.)	Not determined

Macroscopic, Single channel and synaptic current data acquisition and analysis

5   Single channel data acquisition and analysis was performed with an Axon Instruments system using Axobasic and PCLAMP 6.0 software. Additional programs were specifically designed for resolution of multiple channel classes of similar size and kinetics. Conductance, kinetics, NP0 and
10   mean I analyses were performed as previously described (Listerud et al., 1991; Moss and Role, 1993; Moss et al., 1989; Simmons et al., 1988). Continuously recorded and evoked synaptic and macroscopic currents were stored on videotape and analyzed off line in software written in
15   Axobasic.

An 80486 DX2-66Mhz computer equipped with the Axobasic system was essential to all studies. The acquisition program sampled all events that conformed to the amplitude
20   and rise-time criteria, both set up by the user. Each captured trace included 20 msec of pre-event baseline data. The system sampled events accurately up to 20 Hz--entirely adequate for capturing the relatively low frequency events in the experiments described herein. The analysis software
25   provides amplitude, frequency, rise- and decay-time constant information for each current recorded.

Subsequent generation of histograms, cumulative plots fitting, and statistical analyses were performed with
30   Microsoft Excel 3.0, Sigmaplot 4.1 (Jandel Scientific) and Systat. Synaptic current frequency information was divided

into bins for plotting and statistical comparisons. Statistical analyses of differences between control and treatment groups were evaluated by a two-tailed test (Snedecor and Cochran, 1989). Synaptic current amplitude data were compared by plotting cumulative histograms. These plots were also utilized as estimated cumulative probability distributions for the determination of statistically significant differences between treatment groups using the Komolgorov-Simirnov test (Press et al., 1986).

Table II provides a comparison of synaptogenesis vs. recombinant nARIA or ARIA treatment in regulating nAChR gene expression. (Bold serves to highlight the differences.)

Table II. Comparison of Synaptogenesis vs. Recombinant nARIA or ARIA treatment in regulating nAChR gene expression.

AChR subunit	α2	α3	α4	α5	α7	ß2	ß4
Development *in vivo*	ND	170%	↓to ND.	500%	1000%	+/-	600%
Presynaptic input *in vitro*	--	240%	--	315%	150%	--	195%
Target contact *in vitro* Heart----- Kidney----	--	↓;60% of control 148%	--	189% 160%	261% 150%	?	no▲ 143%
recombinant N-ARIA	--	**224%**	--	**248%**	**372%**	?	**[0-400%]**
recombinant ARIA	--	↓;90% of control	--	265%	215%	?	no▲

Table III provides a comparison of the regulation of ACh-gated currents induced by input and target vs nARIA and

ARIA.

Table III. Comparison of the Regulation of ACh-Gated Currents Induced by Input & Target vs. nARIA and ARIA.

Presynaptic input *in vitro*		Target Contact *in vitro:*	
SMN	↑1300%	Heart	↓ 30% of control
VMN	↑750%		
Input Cond. Media		Target Cond. Media	
SMN	↑420%	Heart	↓ 50% of control
VMN	↑430%	Kidney	↑ 225%
recombinant nARIA	↑200-400%	recombinant ARIA	↓60% of control

Experimental Methods

Cell Culture

LSG co-cultures with presynaptic input and target. Dissociated embryonic sympathetic neurons from ED10 and ED17 were prepared and maintained *in vitro* as described in Role 1988 with modifications as noted herein. Under these conditions the neurons were devoid of nonneuronal cells and were both adrenergic and cholinoceptive. Innervation of sympathetic neurons by preganglionic microexplants was done according to previously described techniques (Gardette et al. 1991; Hasselmo and Bower, 1993). Assay of target effects on nAChR expression required coculture of atrial micro-explants (ED12) with LSG neurons *in vitro*. Changes in expression of subunit mRNAs were assayed after 3-4 days of coculture by quantitative RT-PCR (see below and Habecker and Landis, 1994).

Patch Clamp Recording

Recording of macroscopic and synaptic currents employed the whole-cell tight seal recording configuration of the patch clamp technique (Hammil et al., 1981). This techniques
5 provided low noise recordings that allowed for resolution of elemental synaptic currents. Fabrication of patch electrodes, pipette and bath solutions were all as previously described (Moss and Role, 1993; Moss et al., 1989). Currents were recorded with an AXOPATCH 200A patch
10 clamp amplifier and stored on videotape with a PCM digitizer (Instrutech VR-10B) for subsequent analysis off line. $I_{P(AChR)}$ is peak current.

Drug Application

15 Drugs and agonists were applied by microperfusion to small groups of cells via a large barreled delivery tube with continuous macroperfusion at 1 ml/min. This approach optimized speed of application (<30 msec), speed of removal and the ease of changing test solutions applied by the same
20 device. A stable perfusion set-up was an essential component of the each recording set up.

Molecular Techniques

25 ## Identification of subunit gene expression by PCR

The profile of subunit gene expression was analyzed by PCR amplification of cDNA using nAChR subunit specific primers according to our previously published techniques (Listerud et al., 1991). Briefly, total RNA was extracted by
30 homogenization of tissue in 4M guanidine thiocyanate buffer followed by centrifugation through a 5.7M CsCl cushion. The isolated RNA was DNAse treated and reverse transcribed using oligo-dT primers. AMV-RT(reverse transcriptase) was used to amplify a fragment encoding the most variable portion of the
35 nAChR subunits, the intracellular loop. The identity of the

amplified products was verified by restriction mapping and/or Southern blotting (Ausubel et al., 1994).

Quantitative RT-PCR

5   Cell contents were collected by aspiration into DEPC containing solution and cDNA was synthesized by addition of random hexamer primers and Superscript₆ reverse transcriptase enzyme (BRL). The cDNA served as template for amplification by primers specific for the various nAChR

10   subunits. The internal standard construct included sequences complimentary to all upstream and downstream primers used with an interposed multicloning site (MCS) linker. Thus, the efficiency of primer annealing to standard and to the cDNA template was equivalent. In order

15   to detect the product of the reaction, trace amounts of isotope labeled nucleotides were added to the reaction mixture. After 23 rounds of amplification, an aliquot of the reaction mixture was removed and further amplified with fresh Taq polymerase and reaction mix. This step was

20   repeated after another 23 cycles. The amplified fragment was separated from the unincorporated nucleotides by electrophoresis and the product was quantitated. The assay provided subunit specific quantification of α2, α3, α4, α5, α7, α8, ß2 and ß4 in individual samples which detected as

25   little as 2 fg of each subunit.

COS cell transfection

Cells were tansfected according to established techniques (Falls et al., 1993). Briefly, pcDNAl-amp containing the

30   nARIA or ARIA cDNA in sense or antisense orientation was introduced into COS cells using lipofectamine₆ (Gibco-BRL) per manufacturer's instructions. Twenty-four (24) hrs after transfection, the cells were washed and incubated in serum-free OPTI-MEM₆ (Gibco-BRL). After 48 hrs, the media was

35   collected, centrifuged to remove debris and then

concentrated 22-X using a Centriprep 10 concentrator (Amicon). Aliquots were stored at -20°C until use.

Tyrosine-phosphorylation assay

5   Cells were treated with L-15 media plus concentrated conditioned media from either sense, antisense, or non-transfected COS cells. After the desired incubation time, the cells were washed and lysed in 1% NP-40 buffer. The lysate was centrifuged and the protein in the supernatant
10   was quantified with a Bradford analysis procedure (BioRad). Protein samples were electrophoresed on a 4% SDS-PAGE gel and electroblotted onto a PBDF membrane (S&S Inc). Then the membrane was probed for phosphotyrosine with a monoclonal antibody 4G10 (UBI), detected with a peroxidase-conjugated
15   anti-mouse IgG2$_b$ antiserum (Boehringer-Mannheim) and visualized using the luminescent ECL. reagents (Amersham).

In situ hybridization

Cell-specific expression of AChR subunit mRNAs were assayed
20   using *in situ* hybridization in tissue sections of spinal preganglionic nuclei with ^{35}S-labeled complementary RNA probes as described (Devay et al., 1994; Ausubel et al, 1994). cDNAs encoding the non-conserved regions between transmembrane-spanning regions, TM3 and TM4, for each
25   subunit have been subcloned into pGEM-3Z plasmids. Antisense riboprobes were transcribed in the presence of ^{35}S-UTP using the Promega transcription kit. Hybridization was assayed by autoradiography, and Nissl staining allowed visualization of the cell bodies. ^{35}S-labeled RNA probes
30   provided good signal resolution with low background due to the high specific activity (cpm/probe molecule) and also due to the relatively low energy of ^{35}S emission. Determination of hybridization involved comparison between parallel assays with antisense cRNA, sense RNA and RNase pretreatment of the

tissue. Non-isotopic labeling protocol utilizing Digoxigenin-11-UTP in place of the ^{35}S-UTP was also used. The protocols were very similar (Bertrand et al., 1991).

5   Antisense oligonucleotide design and experimental protocols
Antisense oligonucleotides for AChR subunits α2, α3, α4, α5 and α7 were targeted to a 15 base sequence spanning the initiation site of each subunit mRNA. The region upstream and including the ATG was divergent among the subunit
10   sequences and in no case included <4 base mismatch with all chick cDNA sequences registered in GCG. Control oligos included missense sequences of identical composition of oligos mutated at 3 of the 15 bases (same GCG ratio). The uptake, metabolism, hybridization and block of subunit
15   expression by oligonucleotides were studied in some detail to determine optimal conditions for specific block. Briefly, neurons were pretreated with an irreversible nAChR ligand (bromoacetylcholine bromide; BAC) and then incubated for 6-48 hrs with 10 μM oligo in heat inactivated medium)
20   (Gardette et al., 1991; Listerud et al., 1991). The d-oligos were taken up and intact 15-mer within the cells was maximal within 6 hrs and still detected up to 48 hrs. α2, α3, α4, α5 and α7 have all been studied with this technique to determine their contribution to medial habenula nucleus
25   (MHN) and LSG somatic nAChRs with reliable functional block by 24-48 hours. Antisense oligonucleotides were also designed to inhibit the expression of nARIA and ARIA. These oligos were directed against the translation start site or sequence within the N-terminal domain since the sequences
30   are maximally divergent in this region. To optimize the antisense mediated block, and minimize the confounding contribution of pre-existent nARIA and ARIA, antisense was introduced just prior to the initial surge in expression of these factors during development (≈ED4 for nARIA; ≈ED8 for
35   ARIA). Treatment of preganglionic tissue of these ages with

the antisense oligos effectively knocked out the expression of these factors and allowed us to ascertain the extent to which each of these factors contributed to developmental changes in nAChR expression.

5

A question remains as to whether ARIA or nARIA is required for regulation of nAChR channels by presynaptic input. This idea is tested by the selective block of nARIA or ARIA synthesis by antisense-mediated deletion. To optimize the

10   antisense treatment, the region containing the presynaptic neurons is removed prior to the initial surge in nARIA and ARIA expression (E4 and E8, cord; E4 septal region). In this manner we may succeed in blocking the major increase in expression, thereby obviating effects of pre-existent nARIA

15   or ARIA. Presynaptic microexplants are treated for 24-48 hrs *in vitro* with antisense oligos targeted to the initiation region of nARIA or ARIA mRNA. Then, nARIA or ARIA activity may be assayed in co-culture with LSG or MHN neurons (assay of nAChR macroscopic and single channel

20   currents as above). The efficacy of antisense constructs is confirmed by quantitative RT-PCR of control and antisense-treated explants for nARIA, ARIA and transcripts unaltered by the treatment (e.g. actin). Specificity of the antisense is evaluated by assay of oligomers, equivalent in size and

25   composition but with 20-25% mismatched bases. Presynaptic properties (e.g. electrical activity, transmitter release/mini amplitude) may be tested to control for other non-specific effects of the antisense.

30   **Comparison of the affinity of nARIA vs ARIA for heparin sulfate proteoglycan.**
MCF7 breast tumor cells were treated with conditioned media from either ARIA or nARIA transiently transfected COS1 cells. Some of the media was prebound with heparin attached

to glass beads. Prior to treatment, the beads were pelleted by centrifugation to remove any heparin associated proteins. The supernatant was used to treat the MCF7 cells and tyrosine phosphorylation of the ARIA/nARIA receptor was

5   analyzed. See Figure 15. It appears that the ARIA is binding to heparin and is removed from the media by centrifugation. The nARIA lane shows a slight drop in signal which is unaffected by increasing concentrations of heparin (from 6 μg - 60 μg). It is possible that this

10   slight drop is an artifact of differential binding between the lanes and thus nARIA does not bind heparin.

Heparin is a component of both the cellular surface and the extracellular matrix. The difference in the binding

15   affinities of nARIA and ARIA for heparin have two implications. (1) Heparin can affect the affinity of the ligand for the receptor as has been previously shown for HB-EGF. In this case, heparin may increase the affinity for the receptor. Accordingly, a given amount of ARIA may have

20   higher affinity and thus more cellular activity and effect than the equivalent amount of nARIA. This implication may extend to other possible effects of the physiological concentration of the ligand. (2) The lack of affinity for heparin may result in greater solubility of nARIA *in vivo*

25   since the molecule will not be bound to the extracellular heparin. This possibility may influence the localization of the ligand effects and the point concentration of the ligand.

30   Conclusions

The series of experiments described herein are some examples illustrating possible uses of nARIA. As discussed above herein, there are many other possible therapeutic,

diagnostic and pharmacologic uses of nARIA. The biological role of nARIA has been shown to be distinct from that of ARIA and from other members of the heregulin/NDF family. Therefore, nARIA may be useful in therapeutic treatments and as a diagnostic tool for abnormal neuronal conditions. In addition, a comparison of the expression levels and activities of the members of the NDF/heregulin/ARIA family may prove to be useful in the characterization and treatment of neuronal disorders and abnormal conditions and neurological developmental questions which are at this time unanswered.

Example 4: The n-ARIA isoform of neuregulin is both necessary and sufficient for the induction of acetylcholine-receptors in neurons.

It is clear that the n-ARIA isoform of neuregulin is both necessary and sufficient for the induction of acetylcholine-receptors (nAChR) in neurons. The induction of nAChR expression normally occurs at specific sites within the CNS and PNS during synapse formation and can be mimicked by presynaptic input or presynaptic-input conditioned media. Definitive evidence has been uncovered showing that the activity of presynaptic input in inducing receptor expression is mediated by n-ARIA. The timing and pattern of expression as well as the primary structure and functional effects of n-ARIA differ importantly from the other neuregulins.

The expression profile of n-ARIA is distinct from that of the Ig-domain containing forms:

The n-ARIA sequence is unique. An extracellular Ig-like motif, common to all other neuregulins, is replaced by a highly conserved cys rich domain (98% identical chick to

human) and linked to a β1 type EGF-like domain.

n-ARIA is the only neuregulin isoform for which expression is restricted to the nervous system (both PCR and Northern
5   analyses).

n-ARIA expression, unlike the Ig containing forms, is apparent at the earliest stages of neuronal differentiation (E2-E3 in chick; PCR and Northern analyses).
10

The development of isoform specific antibodies and the assessment of the distribution of n-ARIA protein in the CNS and PNS has been performed. A polyclonal sheep antibody was raised which is specific to the cysteine rich domain
15   containing isoforms of the "neu"-regulin gene. This antibody was developed against a peptide sequence encoding the hydrophilic portion within the highly conserved domain. The antibody is capable of recognizing both the denatured and natural protein states on solid matrix support (Figure 16).
20   Immunohistochemical studies demonstrate that this antibody recognizes both avian and mammalian homologues of the nARIA protein. Immunohistochemical studies have also demonstrated that:

25   Unlike the Ig containing forms, n-ARIA is largely (but not exclusively) expressed by cholinergic and/or cholinoceptive neurons. n-ARIA expression is prominent in medial septal cholinergic nuclei, basal forebrain, deep cerebellar nuclei, cerebellar Purkinje neurons, and retinal ganglion neurons as
30   well as in cranial, somatic and visceral motor nuclei.

n-ARIA protein appears to be targeted to axons and axon terminals as soon as such projections can be detected either *in vitro* or *in vivo*. n-ARIA immunoreactivity is localized

in motor nerve terminal at the neuromuscular junction and "double" staining indicates that n-ARIA expression aligns with (post synaptic) α bungarotoxin binding, which reveals the distribution of muscle AchRs. n-ARIA immunoreactivity

5   is also detected in axonal terminals and *en passant* synapses on CNS and PNS neurons (Figure 17).

The functional profile of n-ARIA is distinct from that of the Ig-domain containing forms:

10

n-ARIA is more soluble than the Ig-domain containing forms and is unaffected by heparin and less avidly bound by extracellular matrix proteins.

15   The activity of n-ARIA in inducing tyrosine phosphorylation in neurons is more potent, more rapid and more persistent than Ig-domain containing forms.

The induction of specific nAChR subunits and the enhancement

20   of nAChR-currents by presynaptic input are specifically mimicked by recombinant n-ARIA protein. The activity of n-ARIA in inducing receptor expression in neurons is more potent and more persistent than Ig-domain containing forms (Figure 18).

25

The ability to enhance α3 type nAChR subunit gene expression is unique to the n-ARIA isoform.

Most importantly, the induction of specific nAChR subunits

30   and the enhancement of nAChR-currents by presynaptic input are selectively blocked by prior exposure of the input to n-ARIA specific antisense-oligonucleotides (Figure 19) or n-ARIA specific antibodies.

The n-ARIA isoforms

The role of n-ARIA in the differentiation and synaptic function of the septal cholinergic neurons that project to 5 hippocampus and amygdala has been investigated. Specifically, evidence suggests that n-ARIA might collaborate with other growth factors, previously implicated in the differentiated function and perhaps, survival of CNS cholinergic neurons.

10

n-ARIA may induce several parameters of differentiated phenotype in central cholinergic neurons. The effects of n-ARIA ±target-derived growth factors, on ACH synthesis and release have been examined. Transient depletion of n-ARIA 15 (in vitro by antisense or antibody treatments to "knock down" endogenous n-ARIA) has been utilized to initially test for n-ARIA dependent changes cholinergic neurons.

Genomic probes to n-ARIA (i.e. the sequence encoding the 20 crucial cys rich domain) have been developed. The effects of an n-ARIA-selective gene knock out was examined by using homologous recombination with subsequent excision of the selectable marker employing the Cre-LOXP approach. The n-ARIA exon-specific knock-outs are expected to survive 25 embryogenesis as, unlike other neuregulins (including ARIA), expression is confined to the nervous system, allowing determination of the role of n-ARIA in the differentiation, synaptic function and survival of CNS cholinergic neurons.

30 **EXAMPLE: 5**

Products of the neuregulin gene and their receptors, the erbBs,(erbB2, 3 and 4) control multiple critical biological processes. Dysregulation of Neuregulin-erbB signaling is 35 associated with major human cancers, specifically in breast,

ovarian, prostate and lung cancer. In some patients this is accompanied by the appearance of soluble erbBs in the circulation and/or elevated levels of auto-antibodies recognizing the erbBs. If soluble erbBs are predictive of

5   dysregulated erbB-neuregulin expression or function, then sensitive assays for these proteins in patients' blood has potential to provide non-invasive prognostic information on disease status, both in detecting primary disease and in monitoring disease-free status following traditional

10   therapies. Recently we demonstrated that interactions between neuregulin and erbB expressing cells induces membrane to nuclear translocation of the cytoplasmic domains of neuregulin. We constructed a neuregulin-green fluorescent protein fusion protein that allows us to detect

15   erbB-neuregulin interactions. By coupling this assay with fluorescence activated cell sorting, we are devising a sensitive rapid and high throughput assay for the presence of erbBs in biological fluids. A further modification of this assay detects nuclear targeting of a neuregulin-Gal4-

20   VP16 fusion protein by measuring the expression of a Gal4 regulated reporter gene. The level of reporter gene expression is dependent on the concentration of extracellular stimuli (e.g. erbBs), providing a means of not only detecting soluble erbBs in biological fluids, but

25   quantifying levels. In addition, using reporter gene expression provides an amplification step that significantly increases the sensitivity of the assay.

ROLE OF HEN1 in NEUROGENESIS and RECENT DATA on NEUREGULIN

30

We found tht an embryonic neuronal specific basic-helix-loop-helix protein, HEN1 (also known as NSCL1 or NHLH), interacts with "LIM only" proteins. Examination of the expression patterns of XHEN1 and XLMO-3, the Xenopus

35   homologs of these human genes, reveals extensive overlap

during early neurogenesis. Co-expression of these two genes in Xenopus embryos induces a cascade of expression of neuronal specific basic-helix-loop-helix proteins that leads to neuronal differentiation. Recently, I tested whether

5   neuregulin (NRG) cytoplasmic domain might mediate "back signaling". An intense subnuclear localization was observed in cells transfected with the NRG cytoplasmic domain. A similar observation was made in primary neuron immunostained with an antibody against the cytoplasmic tail of NFRG. This

10 nuclear translocation requires a NLS motif at the beginning of the cytoplasmic domain, which includes eight amino acids: KTKKQRKK.   One   of   the   functions   of   this   nuclear translocation was to induce apoptosis. Furthermore, a novel gene (CNIP) was found to bind to the cytoplasmic domain of

15 NRG, which might be the modulator for the functions of NRG cytoplasmic domain.

CNIP: A NOVEL INTERACTOR PROTEIN SPECIFIC FOR THE CYTOPLASMIC DOMAIN OF CRD NEUREGULIN

20

The neuregulin (NRG) gene encodes a number of splice variants that are epidermal growth factor (EGF)-related polypeptides. The protein structure of most NRG isoforms includes 3 general domains:

25   1.   The variable extracellular domain, including the EGF-like domain but differing amongst NRG isoforms by the inclusion of a cysteine rich or an Ig-like domain (referred to as CRD- or Ig-NRG, respectively,

            2. A conserved transmembrane domain and

30   3. A cytoplasmic domain of unknown function.

We have begun to explore whether the cytoplasmic domain of CRD NRG (also known as nARIA or neuregulin) might mediate "back signaling" by interaction with specific cellular proteins. The interaction "hunt" for the cytoplasmic domain

of NRG was performed using a *Xenopus* cDNA library. cDNAs from the strongest 14 of 400 positives obtained were analyzed. All 14 interactors encode the same product. To test the specificity of the putative interactor, a panel of
5   control baits were screened for binding with the gene product: only LexA-CRD-NRG cytoplasmic domain interacted strongly. Interactions with the cytoplasmic domains of TGFβ receptor, G proteins, cyclin, cyclin dependent kinase or Myc proteins do not occur. The expression pattern of the CRD-
10   NRG Interactor Protein (CNIP) was examined in *Xenopus* and found to be strikingly similar to that of CRD-NRG. Thus CNIP is prominently expressed in the nervous system, especially in the hindbrain, eyes and spinal cord. In view of the specificity of their interactions as well as their
15   overlapping patterns of expression with the nervous system CNIP-NRG interactions may occur *in vivo*.

NOVEL FUNCTIONS OF THE CYTOPLASMIC DOMAIN OF NEUREGULIN

20   Neuregulins (NRGs) comprise a large family of EGF-like growth factors expressed in both the CNS and PNS. The NRG 1 gene encodes multiple splice variants including secreted and transmembrane isoforms. The external (N-terminal) portion of both membrane anchored and secreted NRG isoforms
25   includes a characteristic Ig-like or cysteine rich domain, and an EGF-like domain that is essential for NRG-ErbB interactions. Membrane anchored and secreted NRG isoforms identified to date include one of three distinct (a, b or c-type) cytoplasmic domains. Although the cytoplasmic domains
30   are highly conserved (85% identity form chick to human), the biological function is unknown.

We tested whether NRG cytoplasmic domain(s) might mediate "back signaling" in NRF expressing cells, following

interaction of the tethered ligand with erbB receptors. And intense subnuclear localization was observed in cells transfected with the NRG cytoplasmic domain. A similar observation was made in primary neuron immunostained with an

5 antibody against the cytoplasmic tail of NRG. This nuclear translocation requires a NLS motif at the beginning of the cytoplasmic domain, which includes eight amino acids: KTKKQRKK. The motif is highly conserved (the same sequence for human, rat, mouse, chick and Xenopus NRG). The nuclear

10 translocation was augmented by activation of PKC, or cells expressing erbB receptors. One of the functions of this nuclear translocation was to induce apoptosis. Furthermore, a novel gene (CNIP) was found to bind to the cytoplasmic domain of NRG, which might be the modulator for the

15 functions of NRG cytoplasmic domain.

CRD-NRG IN MOUSE PERIPHERAL NERVOUS SYSTEM DEVELOPMENT.

Synaptogenesis at nerve-muscle junctions involves the

20 redistribution of preexisting surface acetylcholine receptors (AchRs), as well as increased local synthesis and insertion of new receptors. The latter increase in nAChR expression is induced by members of the neuregulin (NRG) family (Fischbach and colleagues *Cell,* 72:801;1993). We

25 have characterized herein an NRG isoform in chicks, mice and humans in which a highly conserve cysteine-rich domain replaces the Ig-motif found in other NRGs; we have termed this variant CRD-NRG. CRD-NRG appears to be both a necessary and sufficient signal for the control of neuronal

30 nAChR expression during synaptogenesis (Neuron, 20:255;1998). In situ hybridization analysis in embryonic mice reveals that CRD-NRG is highly expressed in all cranial nerve nuclei, visceral and somatic motor neurons, and in numerous CNS regions, including the olfactory and

vomeronasal sensory neurons, the main and accessory olfactory and vomeronasal sensory neurons, the main and accessory olfactory bulbs, hippocampus, amygdala, thalamus, and hypothalamus, as well as septal cholinergic and
5   dopaminergic nuclei, we now evaluate the role of NRG1 signaling in the development of the peripheral nervous system using CRD-NRG specific knock out mice by in situ, immunocytological, and immunohistochemical studies.

10   Neuregulins, originally referred to as ARIA, heregulin (HRG), neu differentiation factor (NDF), glial growth factor (GGF), and sensorimotor-derived factor (SMDF), constiture a large family of structurally related glycoproteins which are produce as a consequence of alternative splicing of the
15   *neuregulin-1* (NRG-1) gene.

Neuregulins function as ligands for the erbB family of receptor tyrosine kinases. Targeted disruptions of the entire NRG-1 gene, and of the genes for its three receptors,
20   erb2, erb3, and erb4 have been generated (Gassmann et al., 1995, Lee et al. 1995, Meyer and Birchmeter, 1995, Kramer et al. 1996, Riethmacher et al. 1997, and Erickson et al., 1997). The phenotype of these mice demonstrated the multiple essential roles of this signaling system in the formation of
25   the peripheral nervous system and the heart.

As many as 14 different neuregulin cDNAs have been isolated (Fischback and Rose, 1997). While all known NRG isoforms contain an EGF-like repeat which is considered essential for
30   NRF-1 activity, isoforms may or may not have a kringle domain, an Ig-like domain, a spacer domain, a transmembrane domain, and/or a variety of different cytoplasmic tails. However, all isofoms can be broadly classified into two currently mutually exclusive categories: isoforms which

contain an If-like domain N-terminal to the EGF-like domain (Ig-NRGs), such as ARIA, HRG, NDF, and GGF, and isoforms which contain a cysteine-rich domain N-terminal to the EGF-like domain (CRD-NRFs), such as SMDF.

Different classes of isoforms show distinct patterns of spatial and temporal expression during embryogenesis (Meyer et al. 1997), suggesting that the different isoforms could be mediating distinct biological signals. Analysis of mice null for Ig-containing NRGs and comparative analysis of mice null for all NRGs or just Ig-containing NRFs provides further evidence to support this conclusion (Meyer et al. 1997, Sandrock et al, 1997).

We have found by in situ hybridization analysis in embryonic mice that CRD-NRG is highly expressed in all cranial nerve nuclei, visceral and somatic motor neurons, and in numerous CNS regions, including the olfactory and vomeronasal sensory organs, the main and accessory olfactory bulbs, hippocampus, amygdala, thalamus, and hypothalamus, as well as septal cholinergic and dopaminergic nuclei. In addition, we have generated mice which are homozygous null exclusively for all CRD-containing NRG-1 isoforms. We now present evidence for distinct and essential roles of CRD-mediated NRG-1 signaling in the development of the peripheral and central nervous systems.

From our research over the last year, it is now clear that the n-ARIA isoform of neuregulin is both necessary and sufficient for the induction of acetylcholine-receptors (nAChR) in neurons. As we have previously demonstrated, the induction of nAChR expression normally occurs at specific sites within the CNS and PNS during synapse formation and can be mimicked by presynaptic input or presynaptic-input conditioned media. We now have definitive evidence that the activity of presynaptic input in inducing receptor expression is mediated by **n-ARIA**. The timing and pattern of expression as well as the primary structure and functional effects of **n-ARIA** differ importantly from the other neuregulins.

The expression profile of **n-ARIA** is distinct from that of the Ig-domain containing forms:

As we have previously shown, the n-ARIA sequence is unique. An extracellular Ig-like motif, common to all other neuregulins, is replaced by a highly conserved cys rich domain (98% identical chick to human) and linked to a β1 type EGF-like domain.

n-ARIA is the only neuregulin isoform for which expression is restricted to the nervous system (both PCR and Northern analyses)

n-ARIA expression, unlike the Ig containing forms, is apparent at the earliest stages of neuronal differentiation (E2-3 in chick; PCR and Northern analyses)

A considerable effort in the last year has been in the development of isoform specific antibodies and in the assessment of the distribution of n-ARIA protein in the CNS and PNS. We raised a polyclonal sheep antibody which is specific to the cysteine rich domain containing isoforms of the "neu"-regulin gene. This antibody was developed against a peptide sequence encoding the hydrophilic portion within the highly conserved domain. The antibody is capable of recognizing both the denatured and natural protein states on solid matrix support (Figure 1). Immunohistochemical studies demonstrate that this antibody recognizes both avian and mammalian homologues of the nARIA protein. Immunohistochemical studies have also demonstrated that:

Unlike the Ig containing forms, n-ARIA is largely (but not exclusively) expressed by cholinergic and/or cholinoceptive neurons. n-ARIA expression is prominent in medial septal cholinergic nuclei, basal forebrain, deep cerebellar nuclei, cerebellar purkinje neurons and retinal ganglion neurons as well as in cranial, somatic and visceral motor nuclei.

n-ARIA protein appears to be targeted to axons and axon terminals as soon as such projections can be detected either in vitro or in vivo. n-ARIA immunoreactivity is localized in motor nerve terminals at the neuromuscular junction and "double" staining indicates that n-ARIA expression aligns with (post synaptic) α-bungarotoxin binding, which reveals the distribution of muscle AChRs. n-ARIA immunoreactivity is also detected in axonal terminals and en passant synapses on CNS and PNS neurons (Figure 2)

The functional profile of **n-ARIA** is distinct from that of the Ig-domain containing forms:

n-ARIA is more soluble than the Ig-domain containing forms, and is unaffected by heparin and less avidly bound by extracellular matrix proteins

The activity of **n-ARIA** in inducing tyrosine phosphorylation in neurons is more potent, more rapid and more persistent than Ig-domain containing forms

The induction of specific nAChR subunits and the enhancement of nAChR-currents by presynaptic input are specifically mimicked by recombinant n-ARIA protein. The activity of **n-ARIA** in inducing receptor expression in neurons is more potent and more persistent than Ig-domain containing forms (Figure 3).

The ability to enhance α3 type nAChR subunit gene expression is unique to the **n-ARIA** isoform.

Most importantly, the induction of specific nAChR subunits and the enhancement of nAChR-currents by presynaptic input are selectively blocked by prior exposure of the input to **n-ARIA** specific antisense-oligonucleotides (Figure 4) or **n-ARIA** specific antibodies (in progress).

~~On-going studies on the n-ARIA isoforms~~

Our on-going studies focus on the role of **n-ARIA** in the differentiation and synaptic function of the septal cholinergic neurons that project to hippocampus and amygdala. Specifically, preliminary evidence suggests that n-ARIA might collaborate with other growth factors, previously implicated in the differentiated function and perhaps, survival of CNS cholinergic neurons

n-ARIA may induce several parameters of differentiated phenotype in central cholinergic neurons. We are examining the effects of **n-ARIA** ± target-derived growth factors, on ACh synthesis and release. We will utilize transient depletion of **n-ARIA** (*in vitro* by antisense or antibody treatments to "knock down" endogenous **n-ARIA**) to initially test for **n-ARIA** dependent changes cholinergic neurons.

We are currently developing genomic probes to **n-ARIA** (i.e., the sequence encoding the crucial cys rich domain) we will examine the effects of an n-ARIA-selective gene knock out, by using homologous recombination with subsequent excision of the selectable marker employing the Cre-LOXP approach. The **n-ARIA** exon-specific knock-outs are expected to survive embryogenesis as, unlike other neuregulins (including ARIA) expression is confined to the nervous system, allowing us to determine the role of **n-ARIA** in the differentiation, synaptic function and survival of CNS cholinergic neurons.

Nucleic Acid Sequence of and Protein coded by clone HNC1

This is a clone which screened positive for the human CRD domain of neuregulin.
The insert is subcloned into pBlueScript II KS+ using the EcoR1 site. The
sequence below is a composite of multiple overlapping sequences obtained with
the use of primers designed from the sequence of human neuregulin.

```
            v           v           v           v           v           v           v
                                                                        GAATTC              6

       v           v           v           v           v           v           v
CGGCCAGACATGTCTGAGGTCGCCGCCGAGAGGTCCTCCAGCCCCTCCACTCAGCTGAGTGCAGACCCAT              76
R  P  D  M  S  E  V  A  A  E  R  S  S  S  P  S  T  Q  L  S  A  D  P  S               24

       v           v           v           v           v           v           v
CTCTTGATGGGCTTCCGGCAGCAGAAGACATGCCAGAGCCCCAGACTGAAGATGGGAGAACCCCTGGACT             146
   L  D  G  L  P  A  A  E  D  M  P  E  P  Q  T  E  D  G  R  T  P  G  L               47

       v           v           v           v           v           v           v
CGTGGGCCTGGCCGTGCCCTGCTGTGCCTGCCTAGAAGCTGAGCGCCTGAGAGGTTGCCTCAACTCAGAG            216
   V  G  L  A  V  P  C  C  A  C  L  E  A  E  R  L  R  G  C  L  N  S  E               70

       v           v           v           v           v           v           v
AAAATCTGCATTGTCCCCATCCTGGCTTGCTGGTCAGCCTCTGCCTCTGCATCGCCGGCCTCAAGTGGG            286
K  I  C  I  V  P  I  L  A  C  L  V  S  L  C  L  C  I  A  G  L  K  W  V                94

       v           v           v           v           v           v           v
TATTTGTGGACAAGATCTTTGAATATGACTCTCCTACTCACCTTGACCCTGGGGGGGTTAGGCCAGGACCC           356
   F  V  D  K  I  F  E  Y  D  S  P  T  H  L  D  P  G  G  L  G  Q  D  P             117

       v           v           v           v           v           v           v
TATTATTTCTCTGGACGCAACTGCTGCCTCAGCTGTGTGGGTGTCGTCTGAGGCATACACTTCACCTGTC           426
   I  I  S  L  D  A  T  A  A  C  A  V  W  V  S  S  E  A  Y  T  S  P  V             140

       v           v           v           v           v           v           v
TCTAGGGCTCAATCTGAAAGTGAGGTTCAAATTTCAGTGCAAGGTGACAAGGCTGTTGTCTCCTTTGAAC           496
S  R  A  Q  S  E  S  E  V  L  V  Q  G  D  K  A  V  V  S  F  E  F               164

       v           v           v           v           v           v           v
CATCAGCGGCACCGACACCGAAGAATGTATTTTTGCCTTTTCTTTCTTGCCGTCCACTGCGCCATCCTT           566
   S  A  A  P  T  P  K  N  V  F  F  A  F  S  F  L  P  S  T  A  P  S  F           187

       v           v           v           v           v           v           v
CCCTTCACCCACCCGGAACCCTGAGGTGAGAACGCCCAAGTCAGCAACTCAGCCACAAACAACAGAAACT           636
   P  S  P  T  R  N  P  E  V  P  T  P  K  S  A  T  Q  P  Q  T  T  E  T           210

       v           v           v           v           v           v           v
AATCTCCAAACTGCTCCTAAACTTTCTACATCTACATCCACCACTGGGACAAGCCATCTTGTAAAATGTG           706
N  L  Q  T  A  P  K  L  S  T  S  T  S  T  T  G  T  S  H  L  V  K  C  A            234

       v           v           v           v           v           v           v
CGGAGAAGGAGAAAACTTTCTGTGTGAATGGAGGGGAGTGCTTCATGGTGAAAGACCTTTCAAACCCCTC           776
   E  K  E  K  T  F  C  V  N  G  G  E  C  F  M  V  K  D  L  S  N  P  S           257

       v           v           v           v           v           v           v
TAGTTACTTGTGCAAGTGCCCAAATGAGTTTACTGGTGATCGCTGCCAAAACTACGTAATGGCCAGCTTC           846
```

R Y L C K C P N E F S G R R C Q N Y V M A S F   280

```
               v           v           v           v           v           v           v
TACAAGCATCTTGGGATTGAATTTATGGAGGCGGAGGAGCTGTACCAGAAGAGAGTGCTGACCATAACCG        916
 Y K H L G I E F M E A E E L Y Q K R V L T I T G                                304

               v           v           v           v           v           v           v
GCATCTGCATCGCCCTCCTTGTGGTCGGCATCATGTGTGTGGTGGCCTACTGCAAAACCAAGAAACAGCG        986
   I C I A L L V V G I M C V V A Y C K T K K Q R                                327

               v           v           v           v           v           v           v
GAAAAAGCTGCATGACCGTCTTCGGCAGAGCCTTCGGTCTGAACGAAACAATATGATGAACATTGCCAAT        1056
   K K L H D R L R Q S L R S E R N M M N I A N                                  350

               v           v           v           v           v           v           v
GGGCCTCACCATCCTAACCCACCCCCCGAGAATGTCCAGCTGGTGAATCAATACGTATCTAAAAACGTCA       1126
 G P H H P N P P P E N V Q L V N Q Y V S K N V I                                374

               v           v           v           v           v           v           v
TGTCCAGTGAGCATATTGTTGAGAGAGAAGCAGAGCATCCTTTTCCACCAGTCACTATACTTCCACAGC       1196
   S S E H I V E R E A E T S F S T S H Y T S T A                                397

               v           v           v           v           v           v           v
CCATCACTCCACTACTGTCACCCAGACTCCTAGCCACAGCTGGAGCAACGGACACACTGAAAGCATCCTT      1266
   H H S T T V T Q T P S H S W S N G H T E S I L                                420

               v           v           v           v           v           v           v
TCCGAAAGCCACTCTGTAATCGTGATGTCATCCTAGAAAACAGTAGGCACAGCAGCCCAACTGGGGGCC       1336
 S E S H S V I V M S S V E N S R H S S P T G G P                                444

               v           v           v           v           v           v           v
CAAGAGGACGTCTTAATGGCACAGGAGGCCCTCGTGAATGTAACAGCTTCCTCAGGCATGCCAGAGAAAC      1406
   R G R L N G T G G P F E C N S F L R H A R E T                                467

               v           v           v           v           v           v
CCGTGATTCCTACCGAGACTCTCCTGATAGTCGGATTG     1446
   P V I P T E T L L I V G L                  477
```

(handwritten top) 12

(handwritten) mouse (RD) exon : nucleotides (ats)

(handwritten right margin) go from "start" (p2) to "stop" p(4) rest of sequence is genomic DNA

```
          441         450         459         468         477         486
      CAC CAG CTT CAG ACG CTT GAG GTG AGA AAC ATG CCT TTC AGT TTG GGA TAC TGG
      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
       H   Q   L   Q   T   L   E   V   R   N   M   P   F   S   L   G   Y   W
         T   S   F   R   R   L   R   *   E   T   C   L   S   V   W   D   T   G
         P   A   S   D   A   *   G   E   K   H   A   F   Q   F   G   I   L   V

          495         504         513         522         531         540
      TTT ACT TAA TCA GCT AGG CAT CAG CTT GCT TCC TCT TTT GGT CCC CTG CCT TCT
      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
       F   T   *   S   A   R   H   Q   L   A   S   S   F   G   P   L   P   S
         L   L   N   Q   L   G   I   S   L   L   P   L   L   V   P   C   L   L
         Y   L   I   S   *   A   S   A   C   F   L   F   W   S   P   A   F   L

          549         558         567         576         585         594
      TGA ACC AAC CGG GAT GGT TTG GAG AAG CCT TTG AAA GAA CTG AAA AAG TGT CCC
      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
       *   T   N   R   D   G   L   E   K   P   L   K   E   L   K   K   C   P
         E   P   T   G   M   V   W   R   S   L   *   K   N   *   K   S   V   P
         N   Q   P   G   W   F   G   E   A   F   E   R   T   E   K   V   S   Q

          603         612         621         630         639         648
      AGA AAC AAC AGC TCA AGA TAT TTC GGT ACA CTT CTA TTT CAT AGT TGC TAG AAG
      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
       R   N   N   S   S   R   Y   F   G   T   L   L   F   H   S   C   *   K
         E   T   T   A   Q   D   I   S   V   H   F   Y   F   I   V   A   R   S
         K   Q   Q   L   K   I   F   R   Y   T   S   I   S   *   L   L   E   A

          657         666         675         684         693         702
      CCC CTT CTT TTT CGT TTC TTT TTC TTT TTC TTT TTC TTT TTT TTC TTT TTT TTT
      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
       P   L   L   F   R   F   F   F   F   F   F   F   F   F   F   F   F   F
         P   F   F   F   V   S   F   S   F   S   F   S   F   F   S   F   F   F
         P   S   F   S   F   L   F   L   F   L   F   L   F   F   L   F   F   F

          711         720         729         738         747         756
      TTT TTA TCT TTT TCT TGC TTC CTC TTA AGC TCT CTT ACT TTG GAN AAT GGC CTT
      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
       F   L   S   F   S   C   F   L   L   S   S   L   T   L   K   N   G   L
         F   Y   L   F   L   A   S   S   *   A   L   L   L   W   X   M   A   L
         F   I   F   F   L   L   P   L   K   L   S   Y   F   G   X   W   P   W
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          765         774         783         792         801         810
      GGA NTT GGG TGC CTT ATC GAT TTC CCC CTT CAA GAT GCT GTA TCA TTT GGT TGG
      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
       G   X   G   C   L   I   D   F   P   L   Q   D   A   V   S   F   G   W
         X   L   G   A   L   S   I   S   P   F   K   M   L   Y   H   L   V   G
         X   W   V   P   Y   R   F   P   P   S   R   C   C   I   I   W   L   G
```

(handwritten right margin) ← DNA
(handwritten right margin) } amino acids
(handwritten left margin) from sequence

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          819         828         837         846         855         864
      GGG GAG CTC TGC ATG GTA ATG CAC TGT GAG AGA GGC CGG GCT TTC TGG AGG TGA
      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
       G   E   L   C   M   V   M   H   C   E   R   G   R   A   F   W   R   *
         G   S   S   A   W   *   C   T   V   R   E   A   G   P   S   G   G   D
         G   A   L   H   I   G   N   A   L   *   E   R   P   G   L   L   E   V

          873         882         891         900         909         918
      TCC GGA TGG AGA TTT ATC CCC CAG ACA TGT CTG AGG GAG CTG GCG GGA GGT CCT
      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
       S   G   W   R   F   I   P   Q   T   C   L   R   E   L   A   G   G   P
         P   D   G   D   L   S   P   R   H   V   *   G   S   W   R   E   V   L
         R   M   E   I   Y   P   P   D   M   S   E   G   A   G   G   R   S   S
```

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        927         936         945         954         963         972
CCA GCC CCT CCA CTC AGC TGA GTG CAG ACC CAT CTC TCG ATG GGC TTC CGG CAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   A   P   P   L   S   *   V   Q   T   H   L   S   M   G   F   R   Q
   Q   P   L   H   S   A   E   C   R   P   I   S   R   W   A   S   G   S
     S   P   S   T   Q   L   S   A   D   P   S   L   D   G   L   P   A   X

        981         990         999        1008        1017        1026
CGG AAC ATA TGC CAG ACA CCC ACA CAG AAG ATG GGA GAA GCC CTG GAC TCG TGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   N   I   C   Q   T   P   T   Q   K   M   G   E   A   L   D   S   W
   G   T   Y   A   R   H   P   H   R   R   W   E   K   P   W   T   P   G
     E   H   M   P   D   T   H   T   E   D   G   R   S   P   G   L   L   X

       1035        1044        1053        1062        1071        1080
GCC TGG CGG TGC CCT GCT GTG TCT GCC TGG AAG CGG AGC GTC TCA GAG GGT GCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 A   W   R   C   P   A   V   S   A   W   K   R   S   V   S   E   G   A
   P   G   G   A   L   L   C   L   P   G   S   G   A   S   Q   R   V   L
     L   A   V   P   C   C   V   C   L   E   A   E   R   L   R   G   C   F

       1089         1098        1107        1116        1125        1134
TCA ACT CCG AGA AGA TCT GCA TTG TTC CCA TTC TGG CTT GTA TAG TAA GCC TCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   T   P   R   R   S   A   L   F   P   F   W   L   V   *   *   A   S
   Q   L   R   E   D   L   H   C   S   H   S   G   L   Y   S   K   P   L
     N   S   E   K   I   C   I   V   P   I   L   A   C   I   V   S   L   X

       1143        1152        1161        1170        1179        1188
GCC TCT GCA TTG CTG GCC TAA AGT GGG TAT TTG TGG ACA AGA TAT TTG AAT ACG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 A   S   A   L   L   A   *   S   G   Y   L   W   T   R   Y   L   N   T
   P   L   H   C   W   P   K   V   G   I   C   G   Q   D   I   *   I   R
     L   C   I   A   G   L   K   W   X   F   V   D   K   I   F   E   Y   D

       1197        1206        1215        1224        1233        1242
ACT CTC CTA CCC ACC TTG ACC CTG GGG GGT TAG GCC AGG ACC CTG TTA TTT CTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 T   L   L   P   T   L   T   L   G   G   *   A   R   T   L   L   F   L
   L   S   Y   P   P   *   P   W   G   V   R   P   G   P   C   Y   F   S
     S   P   T   H   L   D   P   X   G   X   L   G   Q   D   P   V   I   S   L

       1251        1260        1269        1278        1287        1296
TGG ATC CAA CGG CTG CCT CCG CTG TTT TGG TAT CAT CCG AGG CAT ACA CTT CAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 W   I   Q   R   L   P   P   L   F   W   Y   H   P   R   H   T   L   H
   G   S   N   G   C   L   R   X   F   G   I   I   R   G   I   H   F   T
     D   P   T   A   A   S   A   X   L   V   S   S   E   A   Y   T   S   X

       1305        1314        1323        1332        1341        1350
TTG TCT CTA AGG CTC AGT CTG AAG CTG AGG CTC ATG TTA CAG GGC AAG GTG ACC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 L   S   L   R   L   S   L   K   L   R   L   M   L   Q   G   K   V   T
   C   L   *   G   S   V   *   S   *   G   S   C   Y   R   A   R   *   P
     V   S   K   A   Q   S   E   A   E   A   H   V   T   G   Q   G   D   H

       1359        1368        1377        1386        1395        1404
ATG TCG CTG TGG CCT CTG AAC CTT CCG CAG TAC CCA CCC GGA AGA ACC GGC TGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 M   S   L   W   P   L   N   L   P   Q   Y   P   P   G   R   T   G   C
   C   R   C   G   L   *   T   F   R   S   T   H   P   E   E   P   A   X
     V   A   V   X   S   X   X   S   A   V   P   T   R   K   N   R   X   S
```

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        1413        1422        1431        1440        1449        1458
CTG CTT TTC CTC CCT TAC ACT CCA CTC CAC CGC CCT TCC CTT CTC CAG CTC GGA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
  L   L   F   L   P   Y   T   P   L   H   R   P   S   L   L   Q   L   G
    C   F   S   S   L   T   L   H   S   T   A   L   P   F   S   S   S   D
    A   F   P   P   L   H   S   T   P   P   P   F   P   S   P   A   R   T

        1467        1476        1485        1494        1503        1512
CCC CTG AGG TGA GAA CAC CCA AGT CAG GAA CTC AGC CAC AAA CAA CAG AAA CTA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
  P   L   R   *   E   H   P   S   Q   E   L   S   H   K   Q   Q   K   L
    P   *   G   E   N   T   Q   V   R   N   S   A   T   N   N   R   N   *
    P   E   V   R   T   P   K   S   G   T   Q   P   Q   T   T   E   T   N

        1521        1530        1539        1548        1557        1566
ATC TGC AAA CTG CTC CTA AAC TTT GTA AGT AGT GAG AGA AAG AAA GGC CAT GGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
  I   C   K   L   L   L   N   F   V   S   S   E   R   K   K   G   D   G
    S   A   N   C   S   *   T   L   *   V   V   R   E   R   K   A   M   V
    L   Q   T   A   P   K   L   C   K   *   *   E   K   E   R   R   W   W

        1575        1584        1593        1602        1611        1620
GGC TTA AAA GAA AGG GTG GGA GGG AAC TTC CAG AAG GCA AAC TCT AGA GGG TTA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
  G   L   K   E   R   V   G   G   N   F   Q   K   A   N   S   R   G   L
    A   *   K   K   G   W   E   G   T   S   R   R   Q   T   L   E   G   *
    L   K   R   K   G   G   R   E   L   P   E   G   K   L   *   R   V   S

        1629        1638        1647        1656        1665        1674
GCT TTT TCC TTT GAG TTT GCA TTT TAA CCG GGA GGT TTT ATT TGG TGA ATC ACG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
  A   F   S   F   E   F   A   F   *   P   G   G   F   I   W   *   I   T
    L   F   P   L   S   L   H   F   N   R   E   V   L   F   G   E   S   R
    F   F   L   *   V   C   I   L   T   G   R   F   Y   L   V   N   H   V

        1683        1692        1701        1710        1719        1728
TGA CAG GGA AAA AAA AAC AAA ATA AAA CAA TTT TAT AAA CTC CTA GGA TGA ATC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
  *   Q   G   K   K   N   K   I   K   Q   F   Y   K   L   L   G   *   I
    D   R   E   K   K   T   K   *   N   N   F   I   N   S   *   D   E   S
    T   G   K   K   Q   N   K   I   L   *   T   P   R   M   N   Q

        1737        1746        1755        1764        1773        1782
AGG TCT TTT AAC AGC AAC TCC ATG TAG CCA GGT TGT TAA GTC TGA GGT TGT CAA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
  R   S   F   N   S   N   S   M   *   P   G   C   *   V   *   G   C   Q
    G   L   L   T   A   T   P   C   S   Q   V   V   K   S   E   V   V   N
    V   F   *   Q   Q   L   H   V   A   R   L   L   S   L   R   L   S   I

        1791        1800        1809        1818        1827        1836
TTA AGA AGT ACA AGC ATC TTT GAA GTT GGG CTT TGG ATG TAA GCT AAG GTC AAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
  L   R   S   T   S   I   F   E   V   G   L   W   M   *   A   K   V   N
    *   E   V   Q   A   S   L   K   L   G   F   G   C   K   L   R   S   I
    K   N   K   H   L   *   S   W   A   L   D   V   S   *   G   Q   L

        1845        1854        1863        1872        1881        1890
TGA TTT GCT GTA GTT TTA GAA GTT AAT ATC TGA CTG TCC TTC ATA TTT TAA TAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
  *   F   A   V   V   L   E   V   N   I   *   L   S   F   I   F   *   *
    D   L   L   C   F   *   K   L   I   S   D   C   P   S   Y   F   N   R
    I   C   C   S   F   R   S   *   Y   L   T   V   L   H   I   L   L
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A. SPECIFIC AIMS

Nicotinic acetylcholine receptors (nAChRs) participate in moving muscles, making memories, and reinforcing our most fundamental behaviors. Decline in the levels of functional nAChRs and deterioration of central cholinergic projections have been implicated in aging-related memory deficits. Dramatic reductions in nAChRs and cholinergic neurons parallel the devastation of cognitive function in Alzheimer's disease.[25,34,39,41,49,51,54,72,88,96,100,107,1 15,129,130,132,133,202-205,221]Deciphering the potential role of nAChRs in memory formation and the impact of deficits in nAChRs on cognition, requires fundamental understanding of the mechanisms controlling the functional properties and cellular targeting of these receptors.

Stimulation of cholinergic projections or application of nicotinic agonists elicits fast excitatory currents via *postsynaptic* nAChRs. In addition, the activation of *presynaptic* nAChRs enhances synaptic transmission by increasing transmitter release.[6,22,27,29,31,72,75,95,100,116,159,190,210] Despite the numerous CNS relays shown to be subject to "synaptic tuning" by nAChR activation, neither the developmental changes nor regulatory signals that control the expression, biophysical profile or targeting of CNS nAChRs are well understood. The prior NS29071 award supported the identification, cloning and initial studies of a molecular signal that we now know is essential for the regulated expression of nAChRs in peripheral ganglia. **The current proposal examines the cellular and molecular mechanisms controlling the expression and functional profile of CNS nAChRs by addressing the following questions.**

AIM 1: DO NEURON-NEURON INTERACTIONS REGULATE THE EXPRESSION, FUNCTIONAL PROFILE AND CELLULAR DISTRIBUTION OF CNS nAChRS?

Presynaptic input *and* target contact, coordinately regulate the profile and pattern of nAChRs expressed at developing ganglionic synapses in chick.[6,33,42,43,64,98,123,124,145,215,219] The elaboration of nAChRs at both pre and postsynaptic sites of cholinoceptive synapses in the CNS is likely controlled by similar cellular interactions.[31,32,34,44,81,121,122,159,162,174,190,206,221,223] **Studies in Aim 1 test whether both cholinergic projections and target interactions control the expression and cellular targeting of nAChRs in the CNS.** We compare the biophysical properties and expression profile of nAChRs in pre-synaptic visceral motor (VMN) and medial habenula (MHN) neurons before and after contacting their respective targets. Parallel studies examine how presynaptic input influences the maturation of nAChRs in the somata-dendritic and axonal domains of cholinoceptive neurons within the interpeduncular nucleus (IPN) and amygdala. Aim 1 studies constitute the essential groundwork for Aims **2 & 3** by combining anatomical, biophysical and molecular biological assays of *in vivo* and *in vitro* preparations. These studies determine how neuronal interactions regulate: (a) the overall levels of nAChR expression: (b) the profile of the nAChR subunits and channel subtypes expressed; and (c) the somata-dendritic vs. axonal distribution of nAChRs during CNS synaptogenesis.

AIM 2: IS CRD-NRG REQUIRED FOR SYNAPTOGENESIS-INDUCED CHANGES IN CNS nAChRs?

Studies during the previous award period affirmed our proposal that "CRD" isoforms of neuregulin are essential regulators of nAChR expression in chick PNS.[75] CRD-NRG is neural specific, abundant in pre-ganglionic (VMN) neurons and is required for the input-dependent regulation of postsynaptic nAChRs in developing sympathetic ganglia. **Aim 2 tests the hypothesis that CRD-NRGs are requisite signals for the synaptic induction, maturation and sustained expression of nAChR channels at pre and postsynaptic cholinoceptive sites in the CNS.** CRD-NRG is the predominant neuregulin isoform in the developing CNS with strong expression in brainstem, motor nuclei, and in subsets of midbrain and basal forebrain cholinergic neurons. Proposed studies pursue initial indications that CRD-NRG signaling is fundamental to the establishment of cholinoceptive synapses and in the maturation of pre and postsynaptic nAChRs. We will test whether CRD-NRG *mimics* input or target-induced changes in nAChRs by treatment of "synaptically naive" neurons with recombinant CRD-NRG *in vitro*. We also will determine whether CRD-NRG is *required* for the regulated expression and maturation of nAChR channels following the initial formation of synaptic connections. Proposed *in vitro* studies compare the expression, functional profile and distribution of nAChRs in synaptic co-cultures treated with control or antisense oligonucleotides targeted against CRD-NRG. Physiological studies of neurons from WT vs. CRD-NRG⁻ mice further test if CRD-NRG signaling is essential for the expression of the mature array of nAChRs at cholinoceptive synapses.

AIM 3: WHAT SIGNALING CASCADES ARE ACTIVATED BY CRD-NRG?

A long-range goal is to determine the biochemical mechanisms underlying CRD-NRG effects in the CNS. Preliminary studies indicate that CRD-NRG activates a diverse array of signaling cascades, involving *both* anterograde and retrograde signaling mechanisms.[209,215] Our first goal is to examine the signaling pathways and molecular mechanisms that underlie the anterograde effects of CRD-NRG on neuregulin receptor (erbB) expressing neurons. By extending preliminary findings these studies determine the time course, dose dependence and relative affinity of recombinant, soluble CRD-NRG activation of specific kinase activated transcriptional cascades. We next pursue findings consistent with retrograde signaling by membrane-tethered CRD-NRG. Preliminary studies include our identification of a NRG cytoplasmic domain interactor-protein ("CNIP") and the demonstration that cleavage of CRD-NRG results in nuclear targeting of the cytoplasmic domain. Potential mechanisms of retrograde signaling via CRD-NRG will be tested in cells stably expressing variants of tagged-NRG cytoplasmic domains – CNIP.

B: BACKGROUND AND SIGNIFICANCE

Summary: This proposal examines the role played by synaptic interactions and candidate signals in regulating the induction and maturation of CNS nAChRs. The ultimate goal of this research program is to identify the cellular and molecular determinants that underlie the development, early plasticity, maintenance, and eventual demise of cholinoceptive synapses in the brain. The following is an outline of recent findings related to this application.

Nicotinic receptors and cholinoceptive synapses in CNS function and dysfunction. Recent efforts to dissect the role of central cholinergic projections and nicotinic receptors (nAChRs) in neurodegenerative diseases have unearthed new and intriguing leads, yet these issues remain controversial. In balance, recent reports support the fundamental importance of central cholinergic synapses in short-term, associative and motivational memory.[5,25,41,48,51,53,56,75,84,88-90,96,100,104,129-131,153,177,190,203,205] Several studies document loss of CNS nAChRs and support the potential for nicotine based therapies in Alzheimer's Disease (AD).[25,34,84,89,90,100,104,107,138,153,177,190,202,203,205] Of particular interest are studies demonstrating a direct relationship between the severity of symptoms of AD or Parkinson's disease and the loss of cholinergic and cholinoceptive phenotypes in amygdala, hippocampus and substantia nigra.[1,21,34,39,49,51,54,88,93,96,107,125,129-132,138,142,146,177,194,203,205,220] Dramatic declines in nAChRs in cholinergic neurons of the pedunculopontine and medial septal nuclei are detected in AD specimens.[25,93,130,132,138,177,202] A (rare!) series of nAChRs assays in normal and AD patients led investigators to propose that quantitative assessment of nAChR down-regulation could be an early index of AD and PD neuropathology.[131,132,194] Finally, recent reviews suggest that changes in nAChRs expression and/or in nicotinic responses contribute to the symptoms, if not the etiology, of Alzheimer's Disease![129,138,203] Nevertheless, the importance of CNS nAChRs is not fully accepted. The conflict derives in part, from the classical bias that in the *CNS*, ACh alters excitability primarily via muscarinic, not nicotinic, receptors. The relative paucity of CNS nAChRs and limited demonstrations of direct, nAChR-mediated, synaptic transmission in the brain, continues to fuel the contention that nAChRs play little, if any, role at CNS cholinergic synapses.

Potential clinical relevance. In view of the emergent support for a fundamental role of cholinergic signaling in neurodegenerative diseases, the current proposal will provide insight into the physiological and pathological regulation of central cholinoceptive systems. Neither the cellular nor the molecular signals that control expression or function of CNS nAChRs are known. In fact the most basic experiments on CNS nAChR synaptic physiology have not been done. These basic data are required to confirm and extend prior anatomical and biochemical studies, and to provide the bases for understanding when and where nAChRs participate in cholinergic signaling in the CNS. Without these data, the role of nAChRs in CNS will remain a point of high controversy.

Activation of nAChRs on presynaptic terminals regulates the efficacy of synaptic transmission. Classic studies of nicotine stimulated transmitter release from isolated nerve terminals,[35,73,94,111,151,210,211] and the (often ignored) work on nAChR mediated facilitation at ganglionic[14,28,29,47,117] and neuromuscular synapses[201] have been widely confirmed (reviewed in [109,117]). In fact, few neurotransmitters (including ACh) remain untouched by the nicotinic "tuning" of synaptic transmission. Multiple assays establish that activation of presynaptic nAChRs augments dopamine release in the ventral striatum.[35,59,?] The release of chatachol- and indoleamines within numerous CNS nuclei is enhanced by nicotine.[99,161,?] GABAergic transmission is strongly potentiated by nAChR activation, an effect apparently involving both somata-dendritic and axon terminal nAChRs.[58,60,117] Last, but not least, electrophysiological studies in prefrontal cortex, interpeduncular nucleus and hippocampus document that even glutamate transmission is subject to control by cholinergic activation of presynaptic nAChRs.[2,74,75,115,191,192]

The parsimonious use of so few nAChR channels to control the gain of so many synaptic relays, suddenly clarifies how nicotine self-administration can exert such a diverse array of cognitive and behavioral effects. In view of the now established importance of pre-synaptic nAChRs in the CNS, it is essential to reconsider the role of nAChRs in CNS function and dysfunction in the more general context of "cholinoceptive sites" (whether pre or postsynaptic) rather than cholinergic synapses. In this view both pre and postsynaptic nAChRs are assessed as the substrates for the cholinergic control of synaptic plasticity. In this proposal we will evaluate how the number and the types of both pre and postsynaptic nAChR channels are regulated in the CNS.

Neuronal nAChR complexes: Physiological properties and regulation of expression. Understanding the role of nAChRs in the CNS requires (at least) a brief consideration of their diverse physiological properties. The family of neuronal nAChR subunit genes currently includes 11 members, designated as α- and β-type subunits. The 8 α-type nAChR subunits fall into two classes ([31,116,164] for review). One group, including $\alpha7$, $\alpha8$, and $\alpha9$, are able to form functional, homomeric complexes that differ from heteromeric (α/β) nAChRs in sensitivity to nanomolar α-bungarotoxin (αBgTx)] or MLA (see Progress &[29,31,116,164,172,219] for review). The remaining α-subunits ($\alpha2$-$\alpha6$) require other α and/or β subunits to form functional nAChRs. Expression of various combinations of α and β subunits (i.e. $\alpha x \alpha y \beta$ or $\alpha \beta x \beta y$) yields biophysically and pharmacologically distinct nAChR channels.[22,31,68,100,116,149,164,190] Subtypes differ in their modulation by internal and external Ca, their distinct single channel kinetics (τ) and conductance (γ), and in agonist and antagonist pharmacology (see [22,31,68,100,116,149,164,190] and **Progress**). Neuronal nAChRs are more permeable to Ca than are muscle AChRs with $\alpha7$ homomers having the highest P_{Ca}/P_{Na} (>10;); Ca permeability also differs amongst other neuronal nAChRs.[31,116,126,152,156,172,199,222] Inclusion of $\alpha5$ with other α's and

ß's yields complexes with >2 x the Ca permeability of the comparable α/ß type complex.[67,68,150,219] These biophysical distinctions have direct impact on the efficacy of both presynaptic and postsynaptic nAChRs. High γ, brief τ nAChRs are spatially segregated from low γ, long τ channels at postsynaptic sites on sympathetic ganglion neurons: the synaptic currents that result are clearly distinct in amplitude and duration ([42,64,114,123] and **Progress**). Activation of α7 containing presynaptic nAChRs enhances transmitter release at MHN-IPN synapses for up to 1 hour, ([70,115]) whereas presynaptic α/ß-type nAChRs elicit more transient synaptic facilitation at interneuronal IPN synapses.

The numbers, functions and spatial distribution of nAChRs are highly regulated in developing PNS neurons.[6,7,33,43,64,123,124,158,223] Increased subunit nAChR mRNAs, increased macroscopic nAChR γ, increased number and probability of nAChR channel openings, and increased number of ligand-binding or immunoreactive sites have been reported. Preliminary work and recent studies of developing CNS reveal regional differences and subunit specific changes in nAChR expression concomitant with synaptogenesis.[7,16,18,32,34,44,81,159,174,206,221,223]

Cholinoceptive sites in the Amygdala.

The amygdala is an integral component of the neural circuits important in memory. Selected regions of the amygdala are ravaged in AD, including the subregions that express nAChRs.[54,93,96,16?,178,202,214] The basolateral (BL) and lateral olfactory tract (LOT) nuclei are the most prominent sites where nAChR mRNA and high affinity nicotine- and αBgTx-binding are detected, and these nuclei are the principal recipients of cholinergic input. Shockingly, neither direct nAChR-mediated synaptic transmission nor nicotine-induced synaptic facilitation has been documented in the amygdala. Our preliminary studies reveal robust nicotine-induced enhancement of transmission at intra-amygdala synapses and suggest that presynaptic nAChRs are induced by a neuron-derived, regulatory signal (CRD-NRG; see below). The initial induction and maturation of nAChRs by amygdala neurons will be examined in **Aims 1 & 2.**

Cholinoceptive sites at Medial habenula-Interpeduncular synapses.

The interpeduncular nucleus (IPN) receives the most robust cholinergic input of any subcortical structure in the brain.[212,213] Afferents include the MHN, medial septal cholinergic neurons, the vertical limb of the diagonal band, and the laterodorsal/pedunculopontine nuclear groups. Cholinergic terminals converge on IPN dendrites immediately apposed to other non-cholinergic synaptic boutons, consistent with the renowned effects of nicotine in modulating GABAergic and glutamatergic transmission in the IPN.[97,116,117,127,212,213] Although nicotine elicits direct (somatic) responses in IPN neurons, nAChR-mediated synaptic transmission is not detected in IPN slice or in MHN-IPN co-cultures.[17,115] Nevertheless, as the hub of CNS cholinergic projections and as a critical relay in higher sensory, arousal and reinforcement circuits, the IPN is an important site for testing mechanisms of nAChR regulation. Preliminary studies reveal that innervation increases expression and somata-dendritic nAChR responses. Furthermore, CRD-NRG (expressed by many, if not all, afferents to IPN) induces significant changes in nAChR expression. **Aims 1 & 2** examine pre and postsynaptic nAChRs at MHN-IPN synapses in detail.

Neuregulin / erbB interactions in neural development and synaptic maturation.

The neuregulin 1 (Nrg-1) gene encodes ligands for erbB tyrosine kinases. Differential splicing of primary Nrg-1 transcripts results in at least 15 distinct protein encoding mRNAs. Each of these isoforms is expressed in a unique temporal and tissue specific pattern.[8,19,45,92,114,120,207,209,215,216] Specific NRG isoforms are implicated in neural and glial development and migration.[57,105,106,161,164,198,209,215] Of particular import, specific isoforms NRG are required for the input-dependent induction of neuronal transmitter receptors for glutamate[136] and ACh ([115] **Progress**). **Aim 2** tests whether CRD-NRG is essential for the initial induction, maturation and targeting of pre and postsynaptic nAChRs in the CNS. **Aim 3** initiates studies of NRG-signaling in cholinoceptive neurons. Hence, a brief overview of NRG/erbB interactions. All NRGs contain the "EGF-like" domain required for receptor binding. Most isoforms are synthesized as single transmembrane domain proteins with one of three cytoplasmic domains. Soluble NRGs, generated by proteolytic cleavage in the external juxtamembrane domain, are thought to act in a paracrine or autocrine manner.[8,19,76,102,103,106,171,215] NRGs activate erbB dimers, inducing extensive cytoplasmic domain tyrosine autophosphorylation. The resultant phosphotyrosines (p-Tyr) are bound by numerous signaling proteins containing either SH2 or PTB domains.[10,23,36,37,93,97,9?,137,139,140,147,148,165,187,193,198,200] These SH2/PTB containing proteins in turn activate specific signaling cascades, most of which involve serine/threonine protein kinases that phosphorylate targets in the plasma membrane, cytosol and nucleus of stimulated cells. The exact nature of the signaling complex assembled, and consequently the biological response, is dictated by the particular Tyr residues phosphorylated.

We identified a novel transmembrane NRG isoform that accounts for the presynaptic, nAChR-inducing activity in chick PNS.[215] This NRG isoform is a novel variant containing a highly conserved (92% aa identity, chick vs. human) Cysteine Rich Domain N-terminal to the EGF domain. Disruption of the mouse Nrg-1 gene results in early embryonic lethality due to defective cardiac development[15,92,102,119,120], providing no information on the role of CRD-NRG in the maturation of central cholinoceptive synapses. We have generated a CRD-NRG specific "knock out" mouse which survives until birth, consistent with CRD-NRGs exclusive expression in the nervous system.[209] In vitro studies comparing different NRGs indicate that they have distinct biological effects,[136,215] but do not relate these differences to specific downstream signaling. We begin to address this question in the experiments proposed in **Aim 3**. CRD-NRG transcripts encode transmembrane proteins[19,76,102,103,106,171,215]. Recent data indicates that CRD-NRG acts as both ligand and receptor. Specific cytoplasmic domain interactor proteins have been identified, and NRG can

be induced to rapidly enter the nucleus.[6,9,197] The potential of membrane tethered NRG-1s to "back signal" is an issue that needs to be (and will be, **Aim 3 Part 2**) explored.

C: PROGRESS REPORT / PRELIMINARY STUDIES

1. PERIOD COVERED AND PERSONNEL:

- This report covers work done on NS29071 between February 1995 (05 year) and November 1998 (08 year).
- As per PHS 4/98 instructions. submission of the Personnel Report (Form JJ) will be completed if so requested by the awarding component.

2. WORK COMPLETED SINCE THE PRIOR COMPETITIVE RENEWAL [→: citations noted as [P1-P17] within the following section refer to publications and manuscripts listed at the end of the Progress Report (C-3)].

> #### *Overall summary of previous award.*
> The previous proposal tested the hypothesis that both presynaptic input and postsynaptic target contact regulate the profile of nAChR channels expressed by developing neurons. **Aim 1** proposed studies assessing the relative contribution of input-derived factors vs. synaptic activity in shaping the mature profile of nAChRs. **Aim 2** delineated a parallel set of experiments testing for possible retrograde influences of various non-neural targets in regulating the nAChR profile at antecedent synapses (i.e. somata-dendritic ganglionic nAChRs). Both Aim 1 and Aim 2 studies utilized *in vitro* preparations of peripheral sympathetic ganglion neurons (SyMps) from embryonic chick; the work was to be extended to the CNS as well. if time permitted.

> #### *Overall summary of work completed.*
> Nearly all of the experiments proposed on both input and target dependent regulation of nAChR expression by chick SyMps are completed at this juncture (3.8 years)[P1-4, P5-9]. Studies testing whether CNS nAChRs are similarly regulated by input and/or target interactions were begun in the last year[P9, P13-16]. The results of the latter studies have guided the design of many of the experiments outlined in **Aim 1** of the current proposal.
>
> The previous application delineated experiments testing whether a candidate nerve-derived factor we had just identified (once called nARIA, now referred to as cysteine rich domain -neuregulin: CRD-NRG) is required for the input-dependent regulation of nAChR expression in SyMps. These studies were successful beyond our wildest dreams! As such, we focussed subsequent efforts on defining the role of CRD-NRG in regulating nAChRs at chick PNS synapses. A distillate of these analyses: CRD-NRG is both necessary and sufficient for input-dependent regulation of nAChRs at ganglionic synapses[P5]. We also began basic mechanistic studies of CRD-NRG-erbB interactions. and on potential differences in tethered vs. soluble CRD-NRG signaling[P10-12]. The current application extends this prior work on CRD-NRG. testing whether this factor regulates expression and cellular targeting of CNS nAChRs **(Aim 2)** and analyzing CRD-NRG activated signaling cascades **(Aim 3)**.
>
> Now for the more difficult. and somewhat personal. issues related to the work completed. The quantity (but not quality) of NS29071 papers currently published is. admittedly. less then impressive. One ("hi-pak") paper detailing all of the studies on input dependent regulation of nAChRs (including the cloning of CRD-neuregulin and all molecular and biophysical tests its nAChR regulatory activity). is published in *Neuron*[P1]. An equally compact paper detailing developmental changes in nAChR composition is published in *J. Physiol. (Lond.)*[P2] A (very compressed) summary of all of the studies on target-specific regulation of synaptic nAChRs is "pre-pro" publication (i.e. in final review) in *Nature Neuroscience*[P3]. Two additional papers are in review for *J. Neurophysiol.*. or for *Neuron*[P4-P5]. Nine (!) papers are in preparation (5 are well along[P8-P14] 4 are at more embryonic stages of "in prep"[P15-P17]). in addition. 3 reviews were solicited based on NS29071 studies and are published (in *Neuron. Ann. Review of Physiol.* and in a textbook, published by Wiley Press[P6-8]). **In summary** The number of NS29071-05 to 09 generated papers will total 17. However. I spent the last year (since 1/7/98) coping with cancer and 5 cancer-related surgeries--greatly delaying my bringing our considerable productivity to PRESS.

PROGRESS ON AIM 1 STUDIES. (*McGehee & Role. 1995; Role & Berg, 1996; Yu & Role 1998; Yang et al., 1998; McGehee.et al.. 1998; Devay et al. 1998; Ramirez-Latorre et al.. 1999; Wolpowitz et al..1999 and 6 manuscripts in preparation*) Aim I studies proposed to examine the developmental regulation of nAChRs. focusing on the potential role of presynaptic input in controlling post-synaptic nAChRs. We resolved to test if presynaptic activity and/or anterograde soluble or membrane bound signals are required for the expression of the mature profile of nAChRs. Experiments assayed the impact of presynaptic input on the expression of nAChR-encoding mRNA. the profile of nAChR currents and on the functional contribution of specific subunits to nAChRs complexes.

1. Developmental vs. input-dependent regulation of nAChR subunit mRNA expression & nAChR channels.

a. Regulation of nAChR gene expression by input and target: The levels of ganglionic nAChR mRNA increase concurrent with synaptogenesis *in vivo* (**Table P1-1**). Work completed during the past award period determined the extent to which presynaptic input vs. non-neural target contact. contributes to the observed developmental changes in nAChR subunit gene expression in sympathetic ganglion neurons (SyMps). We measured

subunit mRNAs by quantitative RT-PCR (qPCR) on RNA from single SyMp cells maintained *in vitro* in the presence or

TABLE P1-1	α3	α5	α7	β4
SYMPs ALONE (set to ≡ 1) mRNA/SyMp (fg/100 fg std)	1.2 ± 0.2	0.4 ± .01	0.2 ± .05	0.4 ± 0.1
SyMps + INPUT	4.9	6.6	18	1.6
SyMps + TARGET♥	0.6	6.3	3.0	4.6
SYMPs+ INPUT+ TARGET♥	2.7	10	23	10
in vivo DEVELOPMENT •	2.8	11	21	12

Anterograde (Input) and Retrograde (Target) co regulation of nAChR expression utilize distinct (~ additive) mechanisms. nAChR mRNA were assayed from synaptically naive SyMs (E9 chick) *in vitro*. Conditions indicated & presented as fold change relative to E9 SyMps (≡ 1). n= (from top):49, 51, 17, 31, 6 experiments of each condition. Data are corrected for amplification efficiency & actin standard[P3] ♥Effects of heart + kidney target shown (see Aim 2 Progress). •RNase protection assay of E9 vs E21, corrected for neuron number and actin standard.

absence of presynaptic input (**Aim 1**) and/or non-neural targets[P1,P3,P4] (**Table P1-1**). In the majority of cases, we collected cytosol for the qPCR assays from the same neurons that were subjected to biophysical scrutiny of the expressed nAChR channels.

Table P1-1 summarizes >100 experiments examining the effects of innervation of, *and* target-contact by, SyMps *in vitro*, and compares these data with *in vivo* measures of nAChR subunit gene expression[P1,P3,P4]. *In vitro innervation* qualitatively recapitulated the induction of nAChRs seen during development *in vivo*. Quantitatively however, the induction of α3 was consistently greater with *in vitro* innervation, whereas the increases in α5, α7, and especially β4, were less than seen *in vivo* (**Table P1-1** & [P1,P3,43,101]). The differences in nAChR induction by *in vitro* innervation vs. *in vivo* development appear to be due, at least in part, to the regulation of SyMp nAChRs by non-neural target tissues (**Table P1-1 & Progress on Aim 2**). Thus *in vitro* target interactions blunt the induction of α3 but strongly upregulate the levels of α5, α7 and β4 – i.e. the effects of input plus target are essentially additive. Furthermore, the overall changes in nAChR subunit gene expression elicited by co-ordinate innervation plus target contact *in vitro* were equivalent to the alterations in nAChR expression measured in developing sympathetic ganglia. *The former result implies that the mechanisms underlying input vs. target induced regulation of nAChR expression are distinct.* Indeed, subsequent analyses of the molecular signals involved in regulating nAChR mRNAs and channels revealed that unique pathways mediate neuronal input vs. non-neural, target-dependent effects (see below: **Progress on Aim 2**). In addition, *the recapitulation of developmental changes in nAChRs by co-ordinate innervation and target contact in vitro supports our hypothesis that synaptic partners (not support cells or cell-autonomous mechanisms) are the dominant regulators of transmitter receptor maturation.*

b. Regulation of nAChR channels: Upon finding that innervation of sympathetic neurons *in vitro* elicited significant changes in nAChR gene expression, we pursued macroscopic and single channel studies delineated in **Aim 1** parts 1 & 4. These studies: (i) assessed how alterations in subunit gene expression affected the profile of nAChR channel subtypes, and (ii) dissected the subunit composition of nAChRs before and after innervation. The ACh-elicited macroscopic currents in *in vitro* innervated neurons ranged from 4-10 fold larger than uninnervated controls.[P3,P4] Within cell comparisons of qPCR data and nAChR mediated responses demonstrated a strong, positive correlation between net ACh-conductance (i.e. the integrated current, corrected for cellular capacitance) and the levels of nAChR mRNA/neuron. In addition, the amplitude and kinetics of TTX-resistant, nAChR-mediated synaptic currents (sPSCs)[P3,P4] suggests that *in vitro* innervation, like *in vivo* development, increases the number of large conductance but brief duration (τ₀) nAChRs. The combined results of synaptic current analysis and same-cell qPCR assays of nAChR subunit mRNA, support the proposal[P3,P4] that presynaptic input increases the expression of nAChR complexes that include both α3 and α5 (with β4). Furthermore, presynaptic input strongly induced expression of α7 containing nAChR complexes in postsynaptic (i.e. SyMp) neurons compared with non-innervated controls[P3,P4]. These results are discussed in more depth in **Progress on Aim 2**.

We also completed proposed studies defining changes in the *functional* contribution of the α3, α5 and α7 subunits to SyMp nAChRs.[P2,P1,218,219] We probed the contribution of individual nAChR subunits to the single channel profile of SyMps, before and after innervation, using subunit specific antisense oligonucleotides and selected pharmacological agents. A compendium of the changing array of nAChR subtypes and subunit complexes expressed during ganglionic synaptogenesis was published in back-to-back papers (in J. Physiol. (Lond.)) early in 1998[218,219]. In sum, these studies revealed far greater complexity in the synaptogenesis associated changes in nAChR channels and in their apparent subunit composition than previously proposed. Briefly, we conclude that: **1st**, the participation of α5 and α7 in nAChR complexes steadily increases following innervation. Rather than an abrupt developmental switch to the mature nAChR channel profile, we found that all seven (!) nAChR subtypes were present during synaptogenesis. **2nd**, the functional contribution of α3, α5 and α7 subunits to distinct nAChR channels dictates the agonist and antagonist sensitivity of each subtype, in addition to determining their open time kinetics (τ₀) and opening probability (Po). **3rd**, in conjunction with numerous studies by other laboratories, the data summarized above indicates that: (i) native nAChRs can include mixes of more than one α (or β [30]) subunit, and (ii) native nAChRs have more diverse stoichiometries than the 2α:3β configuration of "CNS-type" α4β2 nAChRs. Finally, our results support the (admittedly controversial) idea that (iii) α7 participates in heteromeric and homomeric nAChR complexes. Our current model of SyMp nAChR subunit composition and the influence of both input and target on the profile of nAChRs, is summarized in the conclusion section of this progress report (and see[218,219] & reprint enclosed). Although (still) "just a model", the proposal now draws on the convergence of single cell qPCR, synaptic current,

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Principal Investigator/Program Director (Last, first, middle): Role: L.W. NS29071

single channel, antisense and pharmacological studies. Details of our model notwithstanding, the essential results of these "background" studies are clear: both presynaptic input and target contact regulate nAChR expression. As such, the proposed examination of the mechanism(s) underlying the regulation of nAChR expression in SyMps by both pre and postsynaptic partners was well worth pursuing.

2. What are the cellular & molecular mechanisms of input-dependent regulation of nAChRs?

Activity: Subsequent studies assessed whether input-dependent regulation of nAChRs was due to synaptic activity and/or input-derived signals. All tests of whether synaptic transmission at visceral motor (VMN)-SyMp synapses regulate nAChRs yielded a resounding "no". Chronically blocking transmission (for 24–48 hr) with hexamethonium, d-TC, mecamylamine, or combinations thereof, had no effect on nAChR subunit expression, channel profile or on the distribution of somata-dendritic nAChRs. With the usual caveats required for interpreting a negative result, we concluded that nicotinic transmission does not regulate nAChR expression.

b. Input-derived "factors": We next tested possible activity-**in**dependent mechanisms of nAChR induction by VMN. We completed the proposed studies, which included: i. tests of VMN-derived soluble factors assayed in transfilter co-cultures or by treatment of synaptically naive SyMps with medium conditioned by VMN explants; ii. tests of VMN-derived membrane-associated factors (i.e. contact-mediated effects); iii. assays of candidate factors, including CRD-NRGs and Ig-NRGs (a.k.a. ARIA, GGFII). The latter studies involved treatment of SyMps with native or recombinant protein and "functional deletion" of candidate regulators from VMN by treatment with antisense oligonucleotides designed to selectively block translation (as in prior studies[8,101,115,150,217-219]). These detailed studies, published in [P1] and updated in **Fig. P1-1**, converge on a simple conclusion: CRD-NRG is a (the?) VMN-derived signal required for expression of the "mature" array of nAChR channels at developing ganglionic synapses. We demonstrated that: i. VMNs regulate the level of expression and the functional profile of SyMp nAChRs by mechanisms involving *both* soluble and contact-dependent factors; ii. CRD-containing NRGs are the predominant isoforms expressed by preganglionic neurons during synaptogenesis; iii. rCRD-NRG induces sustained (> 3 days) upregulation of α3, α5, α7 and β4 mRNA, characteristic of the input-induced increases in nAChR subunit gene expression. Other candidate factors (e.g. Ig-NRGs, LIF, TGFβ) were either without effect or elicited only transient alterations in nAChRs; iv. finally, we completed a critical set of experiments (proposed as "long range studies") that affirmed that CRD-NRG plays a fundamental role in the normal, input-dependent upregulation of nAChRs and "mature" high γ, high Po and brief to nAChR channels. Treatment of VMNs with CRD-NRG-specific antisense oligonucleotides blocked all of the nAChR inducing activity released by VMNs, whereas Ig-NRG AS and several CRD "mismatch" oligonucleotides were without effect. These "functional deletion" studies also revealed a contact-mediated component of the input-dependent regulation of postsynaptic nAChRs. Although postsynaptic nAChR induction was strongly inhibited by CRD-NRG AS treatment of VMNs prior to synapse formation, it was not completely blocked. The persistence of nAChR inducing activity in synaptic co-cultures of VM and SyMp neurons might be due to residual membrane tethered CRD-NRG or to other, NRG-independent mechanisms. Genetic deletion of CRD-NRG should resolve this issue (see below). Particularly intriguing are results of initial assays of the postsynaptic nAChR channels at synapses in co-cultures of CRD-NRG "deleted" VMNs and SyMps. Not only were the unit sPSC small in amplitude (consistent with the reduced postsynaptic n and/or Po), their decay kinetics were abnormally slow (**Fig. P1-1B**, insets), indicative of long to channels underlying the ensemble synaptic currents. Thus as at abnormally low levels of CRD-NRG, presynaptic input may only induce "immature" (low γ, long to) nAChRs. Although speculative, it also is possible that decreased CRD-NRG synthesis during development or aging results in regression to embryonic-type CNS nAChRs (i.e. > to, < γ nAChRs).

c. Progress on the biochemical characterization of CRD-NRG. Sequencing and cDNA expression revealed that CRD-NRG is a non-heparin binding, splice variant of NRG.[3] Attempts to purify CRD-NRG are in progress using a His-tagged-CRD-NRG fusion protein expressed in HEK cells. Small scale, Ni+ affinity chromatography of conditioned media yielded a fraction that contained 2 proteins of ~66 and 55-60 kD (**Fig. P1-2**). Affinity chromatographic and immunological techniques indicated that these proteins are BSA and CRD-NRG respectively, and showed that CRD-NRG retained bioactivity (as assayed by P185 tyrosine phosphorylation; **Fig P1-2**). We are scaling-up the purification and will use purified material in the experiments proposed. Studies using semi-purified, rCRD-NRG, demonstrated that rCRD-NRG has a spectrum of erbB interactions that are distinct from those of the EGF-like peptide of NRG (which behaved nearly identically to rIG-NRG).[P5] 32D cells expressing receptor binding. EGF-like peptide of NRG (which behaved nearly identically to rIG-NRG).[P5] 32D cells expressing single erbBs, or pairwise combinations, were treated with rCRD-NRG or NRG EGF-peptide for 48 h, after which proliferation was assayed by ^{3}H-thymidine incorporation. 32D cells are dependent on IL-3 for proliferation and do not express erbBs or NRG. Expressing erbBs allows erbB ligands (e.g. NRG or EGF) to substitute for IL-3.[P5] The results (**Fig. P1-2B**) demonstrate that NRG EGF-peptide was mitogenic for all cells expressing erbB3 or erbB4 (+/-erbB2). rCRD-NRG stimulated cells expressing erbB4, and stimulated erbB2:3 and erbB2:4 cells as well as, or better than, NRG EGF-peptide. Thus, rCRD-NRG, in contrast to other isoforms, shows a preference for erbB2:B4 dimers.

Examination of the spatial and temporal pattern of CRD-NRG expression was pursued in detail, initially focusing on developing chick PNS and, more recently, on developing mouse PNS and CNS. CRD-NRG is expressed

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Number pages consecutively at the bottom throughout the application. Do not use suffixes such as 3a, 3b.

(measured by mRNA in situ hybridization (ISH) and binding with an CRD-specific antibody, described in [1]) strongly in visceral and somatic motor pools, in cranial sensory and motor nuclei, in brainstem and in midbrain structures that are equivalent to mammalian dorsolateral, ventral tegmental and medial septal nuclei.[1,25,4,8] Particularly striking was the early and selective targeting of CRD-NRG protein to axonal projections (e.g. see [1] Fig.5B, enclosed) supporting the proposed role of CRD-NRG in regulating postsynaptic differentiation. Some of our more recent studies on the expression of CRD-NRG and NRG receptors (erb B2,3 and 4) in mice are presented in **Aim 2**[1,1].

Over the last year of the previous award, we initiated studies of the role of CRD-NRG in the establishment and maturation of cholinoceptive synapses in mammalian PNS and CNS. These studies involved the generation of a mouse in which the CRD-encoding exon of the Nrg-1 gene was selectively disrupted by homologous recombination. The generation of this CRD-NRG "knock out" mouse and the first set of studies characterizing the effects of selective deletion of CRD-NRG at somatic motor synapses, is described in detail in a submitted manuscript[5], included with this application.

Initial studies of central cholinergic projections in general, and of visceral motoneurons in particular, indicate that loss of CRD-NRG results in aborted formation of cholinoceptive synapses and in altered patterns of nAChR expression by both pre and postsynaptic neurons. In CRD-NRG[-] mice, VMNs that normally express CRD-NRG, still extended axons into target sympathetic ganglia, and primary SyMps expressed detectable levels of nAChR subunit mRNAs and channels. However, neither ganglionic nor neuromuscular cholinoceptive synapses were sustained in CRD-NRG[-] mice. By P0, cholinergic axons withdrew and the postsynaptic nAChRs detected remained immature, both in their expression levels and in their spatial expression pattern (**Fig.P1-3** & [P5] Fig.2&3). **Aim 2** studies will examine the role of CRD-NRG in the maturation of neuronal cholinoceptive synapses in detail.

PROGRESS ON AIM 2 STUDIES (Devav et al.,1998 McGehee & Role, 1995; Role & Berg, 1996 and 3 manuscripts in preparation). Prior **Aim 2** studies proposed to examine the developmental regulation of nAChRs, with a focus on the potential role of target interactions and retrograde factors. We resolved first to explore if the non-neural targets of sympathetic ganglia could influence nAChRs expressed at the antecedent (ganglionic) synapse. Subsequent studies were to test if synaptic transmission and/or retrograde soluble or membrane bound signals were required for target-dependent changes in nAChR expression. We also planned studies of target-dependent regulation of CNS nAChRs.

1. Target-dependent regulation of nAChR subunit mRNA expression & nAChR channels.

Target-dependent changes in nAChR expression in uninnervated SyMps This first line of studies of non-neural target dependent changes in ganglionic nAChRs demonstrated that contact of SyMps with renal or cardiac tissue profoundly altered SyMp nAChRs. Furthermore the profile of nAChR subtypes was differentially regulated by the two targets. SyMps were removed prior to innervation *in vivo* and maintained *in vitro* in the presence of renal or cardiac tissue targets and without presynaptic input. Subsequent assays measured the concentration dependence and profile of ACh-elicited macroscopic and single-channel currents, and used qPCR to quantitate the levels of expression of the $\alpha 3$, $\alpha 5$, $\alpha 7$ and $\beta 4$ nAChR mRNAs. The differential effects of the two targets were evident in each assay (**Fig.P2-1** & [P3]).

Briefly, kidney contact increased the amplitude of ACh-evoked macroscopic currents and induced expression of high-[ ] brief-τo nAChR channels that were not detected in SyMps maintained in the absence of target. Consistent with the expression of a large number of relatively low-affinity nAChRs, channel activity showed little decline during patch recordings. Likewise, increases in the ACh concentration beyond what is normally super-maximal, elicited further increases in macroscopic current amplitude in SyMps contacting kidney. In SyMps without target or that contacted heart ACh-elicited macroscopic currents, were significantly smaller than the currents in SyMps co-cultured with kidney. The profile of nAChR single-channel currents recorded in SyMps + cardiocytes was distinct from that in SyMps without target or SyMps contacting renal tissue. Most ACh-gated channels recorded in SyMps innervating heart were low-[ ] long τ_o events (**Fig.P2-1** & [P3]). qPCR analysis of nAChR-subunit mRNAs revealed significant increases in the levels of expression of $\alpha 3$, $\alpha 5$ and $\beta 4$ in all neurons contacting the renal tissue explants. In contrast, cardiac target contact, upregulated $\alpha 5$, $\alpha 7$ and $\beta 4$, but not $\alpha 3$ mRNA (**Table P1-1** & [P3]).

2. Role of synaptic activity vs. target derived signals in the regulation of SyMp nAChR expression.

The observed changes in nAChR expression induced by contact with renal and cardiac tissue appear to involve mechanisms that are distinct from one another and from the input-derived (i.e. CRD-NRG mediated) regulation of nAChRs. Treatment of input- and target-naive SyMps with a membrane fraction prepared from cardiac tissue mimicked the effect of heart co-culture on nAChR expression. In contrast, cardiac-derived soluble factor(s) were without effect (e.g. I_{ACh}, + heart membrane $475 \pm 62pA$, n=18 vs. neurons alone 744 ± 66 pA, n=29).

Neither soluble factors or membrane fractions from renal tissue mimicked the effects of kidney contact. Further analysis of SyMp nAChRs induction by kidney indicated that in this case, synaptic transmission between sympathetic neurons and renal target was required. Concomitant addition of α and β adrenergic receptor antagonists to sympathetic neuron-kidney co-cultures eliminated the inductive effects of kidney on nAChR subunits (e.g. $\alpha 3$ mRNA; SyMps + kidney: $240 \pm 30\%$ of control vs. SyMps + kidney + adrenergic blockers: 105 ± 10 % control, n= 3).

The requirement for adrenergic transmission in the renal induction of neuronal nAChR expression is reminiscent of target-induced changes in the neurotransmitter phenotype of developing sympathetic ganglia. [169]

3. Coordinate target- and input induced changes in ganglionic nAChRs. Our prior Our prior studies implicitly assumed that the final array of nAChRs at mature ganglionic synapses is dictated by the combined influences of pre- and post-synaptic partners. Hence, the "ultimate" experiment requires assessment of the properties of nAChR channels on SyMps that are both innervated and in contact with specific targets. As patch clamp recording of synaptic channels *per se* is difficult (due to the physical barrier of the overlying presynaptic bouton), we conducted a detailed analysis of synaptic currents in SyMps contacting cardiac or renal target. The combined influence of input and target, as well as the specific impact of each target tissue, on nAChRs, was evident in changes in the biophysical profile of synaptic nAChRs and in nAChR expression (Fig P2-2, **Table P1-1, b low &** [69], enclosed). The ability of concomitant innervation and target contact *in vitro*, to recapitulate *in vivo* nAChR expression, indicates that synaptic partners (rather than support cells or cell-autonomous mechanisms) dictate the profile of nAChRs expressed at mature, PNS cholinoceptive synapses.

SUMMARY OF PROGRESS

As originally proposed, we determined the net effects of input *and* target contact on the expression of nAChR subunits and on the properties of nAChR channels in developing chick sympathetic ganglia. We conclude that anterograde and retrograde signals dictate the ultimate array of synaptic nAChRs in the PNS (**Table P1-2**). We identified a candidate presynaptic-derived signal as a novel, neural-specific NRG isoform that is expressed by VMNs prior to ganglionic synaptogenesis. Finally, we demonstrated that this novel, Nrg-1 gene product is essential for the input-dependent maturation nAChR. Studies initiated toward defining the role of CRD-NRG in the regulation of CNS nAChRs include our generation of an isoform specific "knock out" mouse.

TABLE P2-1: Σ Regulation of nAChR s	$(\alpha3)_2(\beta x)_3$	$(\alpha3)_1(\alpha7)_1(\beta x)_3$	$\alpha3)_3(\beta x)_2$	$(\alpha3)_1(\alpha5)_x(\alpha7)_1(\beta x)_2$	$(\alpha3)_2(\alpha5)_2(\beta x)$	$(\alpha3)_1(\alpha5)_2(\alpha7)_1(\beta x)$	$\alpha7$
γ (pS)	13.5 ± 2	23 ± 3	28 ± 4	38 ± 6	50 ± 3	51 ± 3	66 ± 7
τ_O 1 (ms)	2.1 ± 5	1.1 ± 0.2	1.7 ± 0.1	1.7 ± 0.1	3.3 ± 0.5	3.3 ± 0.4	2.5 ± 0.8
τ_O 2 (ms)	7.6 ± 1 (65%)	7.0 ± 1 (60%)	13 ± 0.9	10.8 ± 0.4 (67%)	-	16.6 ± 3.1 (39 %)	-
ABUNDANCE							
Early develop.	++++	-	++	-	+	-	-
Intermediate	+++	+	++	++++	+++	+++	+
Late develop.	-	++	-	++++	++++	++++	++
INDUCED by...							
Input	-	-	-	++	+++	++	+
Contact with kidney	-	-	-	+	++++	-	+++
Contact with heart	++++	++	+	-	-	-	-
PHARMACOLOGY	the number of + 's represents the relative apparent affinity for ACh (where > + s indicates > apparent affinity)						
ACh (rel. K_{app})	++++	+++	++++	++	++	+	+
Cytisine	+	-	+	-	+	-	ND
n-BgTx sensitivity	+	-	+	-	-	+	+
α-BgTx sensitivity	-	-	-	-	-	-	ND
MLA sensitivity	-	-	-	-	-	+	+
DELETED by AS to :	$\alpha3$	$\alpha3, \alpha7$	$\alpha3$	$\alpha3, \alpha5, \alpha7$	$\alpha3, \alpha5$	$\alpha3, \alpha5, \alpha7$	$\alpha7$, **ND**

C3: PUBLISHED PAPERS AND MANUSCRIPTS SUPPORTED BY NS29071

Primary, peer reviewed papers   → [App.] indicates inclusion in the APPENDIX.

1. Yang, X., Kuo, Y., Devay, P., Yu, C., and Role, L. (1998) A cysteine-rich isoform of neuregulin (CRD NRG-1) controls the level of expression of nAChR channels during synaptogenesis. *Neuron*: 20:255-270. [App.]
2. Yu, C. and Role, L. (1998) Functional contribution of the α5 subunit to neuronal nicotinic channels expressed by chick sympathetic ganglion neurones. *J. Physiol* (Lond.) 509: 667-681. [App.]
3. Devay, P., McGehee D.S., Yu, C. and Role, L. W. (1998) Target specific control of nicotine receptor expression at autonomic interneuronal synapses in developing chick. (*Nature Neuroscience*). [App.]
4. McGehee, D., Devay, P., Role, L.W. and Brussaard, A.B. (1998) Innervation-induced changes in neuronal nicotinic ACh receptors increase synaptic efficacy (in review *J. Neurophysiology*). [App.]
5. Wolpowitz, D., Mason, A., Mendelsohn, M., Talmage, D.A. and Role, L. (1999) Requirement for cysteine-rich domain isoforms of the neuregulin 1 gene in the development of PNS synapses (in review *Neuron*). [App.]

Solicited reviews and book chapters covering NS220971-generated results

6. McGehee, D. and Role, L. (1995) Physiological diversity of acetylcholine receptor channels expressed in vertebrate neurons. *Annual Review of Physiology*, 57:521-546. [App.]
7. Role, L. and Berg, D.K. (1996) Nicotinic receptors in the development and modulation of CNS synapses *Neuron*. 16:1077-85 [App.]

3. Ramirez-Latorre, J., Crabtree, G., and Role, L. (1999) Molecular composition and biophysical characteristics of nicotinic receptors. In *Neuronal nicotinic receptors: Pharmacology and Therapeutic Opportunities* (eds., S. P. Arneric, & J. D. Brioni & Wiley-Liss, Inc) in Press.

MANUSCRIPTS OF NS 29071–SPONSORED WORK IN PREPARATION.

9. Yang, X., Wolpowitz, D., Kuo, Y., and Role, L. (1999) Expression of CRD-NRG-1 during CNS and PNS development in chick.

10. Talmage, D.A., Kuo, Y., Wang, L., Pierce, J., Yang, X., and Role, L. (1999) Differential potency and efficacy of neuregulin isoforms in the activation of Her3 vs. Her 4 receptors depends on N-terminal sequence domain.

11. Bao, J., Role, L., Talmage, D.A. (1999) Activation of transmembrane neuregulin isoforms by soluble erbB3/4 or by erbB expressing cell lines, induces nuclear localization of the NRG c form cytoplasmic domain (in prep).

12. Bao, J., Talmage, D.A., Role, L. (1999) CNIP: A novel interactor protein specific for the cytoplasmic domain of CRD neuregulin.

13. Wolpowitz, D., Mason, A., Davis, T., Epstein, D. and Role, L.W. (1999) Expression of CRD-NRG and erbB3 and erbB4 in central and peripheral neurons during development of the mouse.

14. Wolpowitz, D., Mason, A., Epstein, D., Davis, T., Orr-Urtreger, A. and Role, L.W. (1999) Development of cholinergic projections and expression of nAChR $\alpha3,\alpha4,\alpha5,\alpha7$ and $\beta2$ subunits in the developing mouse brain and spinal cord.

15. Girod, R., Jareb, M. and Role, L.W. (1999) Targeting of $\alpha7$-containing nAChRs to presynaptic sites at visceral motor-sympathetic neuron synapses.

16. Du, C., Jareb, M., Devay, P. and Role, L.W. (1999) CRD-neuregulin is required for increased expression and targeting of presynaptic nAChRs at visceral motor-sympathetic ganglion synapses.

17. Barazangi, N. and Role, L. (1999) Nicotine-induced enhancement of excitatory and inhibitory synaptic transmission in mouse amygdala.

D. RESEARCH DESIGN AND METHODS

AIM 1: DO NEURON-NEURON INTERACTIONS REGULATE THE EXPRESSION, FUNCTIONAL PROFILE AND CELLULAR DISTRIBUTION OF CNS nAChRS?

Overall Rationale for Aim 1 Studies:

Neuronal nAChRs play a key role in controlling "synaptic gain" throughout the CNS. Hence, the cellular and molecular mechanisms that control the expression and synaptic targeting of these receptors are fundamentally important. **Aim 1 studies test the hypothesis that interactions with input *and* target neurons are requir d for the maturation of cholinoceptive synapses in the CNS.** The experimental design of Aim 1 was guided by consideration of: 1st. Prior demonstrations that both input and non-neural target interactions regulate peripheral (ganglionic) cholinoceptive sites in chick (see **Background & Progress**) 2nd. Recent reports that the levels of expression, and perhaps the composition, of nAChRs change during the period of synaptogenesis in brain and spinal cord (see **Background**) and 3rd: Our preliminary work toward this renewal application (see& **Progress &** below). We will test if observed "developmental changes" in nAChR expression are specifically induced by pre and postsynaptic partners in the CNS, as they are in the PNS. Proposed experiments employ biophysical and molecular techniques to study both avian and mammalian cholinoceptive synapses. The "leap to mouse", while keeping bird in hand (sorry), reflects the particular advantages of each system: the chick offers relative simplicity, accessibility, and an unparalleled font of background data from decades of scrutiny. Nevertheless, the chick refuses to submit to classic molecular genetic techniques – an undeniable limitation to our interests in developmental *mechanisms*: Hence: the proposed work in mouse.

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Principal Investigator/Program Director (*Last, first, middle*): Role. L.W. NS29071.

Overall Approach to Aim 1 Studies:

More than half of the experiments in Aim 1 utilize primary chick or mouse neurons in synaptic co-cultures. Studies of synaptic maturation *in vitro*, are straightforward – ideal actually, for proposed analyses of pre and postsynaptic nAChRs. We also will pursue our initial studies of CNS cholinoceptive synapses in intact (slice) preparations. Although certainly more complex, the slice studies are essential to understanding now cholinoceptive sites mature *in vivo*. In addition, the brain-slice experiments provide the essential groundwork for studies of mice genetically engineered to lack putative "1st messengers" that control nAChR expression *in vivo* (see **Aim 2**). **Aim 1, Studies 1-3** test for effects of target-neuron contact on two classes of CNS neurons that express nAChRs in both somata-dendritic and axon-terminal domains – the visceral motor (VMN) and medial habenula (MHN) nuclei. **Studies 4-6** examine the development of nAChR-mediated responses at 2 sites of projection of the medial septal, basal forebrain and pedunculopontine cholinergic systems - the IPN and amygdala.

Studies using synaptic co-cultures examine: (a) The number and profile of nAChR channels using macroscopic and single channel current recording; (b) The levels and profile of nAChR subunit gene expression by qPCR; (c) The overall distribution of nAChRs in somata-dendritic vs. axonal domains by agonist-induced changes in calcium and voltage responses, and by mapping the distribution of tagged-nAChRs expressed in primary neurons following adenovirus mediated infection; (d) The segregation and clustering of somata-dendritic nAChRs by repeat single channel "patch mapping" of individual neurons. Finally, in synaptic co-cultures, (e) targeting of nAChRs to axon-terminals or preterminal regions by assays of nAChR-mediated facilitation of spontaneous and evoked transmission. Studies of cholinoceptive synapses in intact slice assess: (a) The profile of the nAChR subunit mRNA expressed in individual neurons; (b) The expression of somata-dendritic nAChRs by macroscopic (perforated patch) recording of nicotinic agonist-elicited responses; and (c) The expression of presynaptic nAChRs by monitoring ACh-induced facilitation of spontaneous and evoked synaptic transmission.

AIM 1: PROPOSED EXPERIMENTS:

Part 1: Does interaction with neuronal targets regulate CNS nAChRs?

*Aim 1 Study 1: **Determine if nAChR gene expression by CNS cholinoceptive neurons is alt red by interactions with their post-synaptic partners**.* The following experiments provide an important molecular gauge to our otherwise electrophysiological analyses of receptor regulation. Although changes in subunit gene expression can not be extrapolated to changes in the profile of functional, surface nAChRs, overall trends and even small changes in levels of gene expression have provided important hints as to possible alterations in the nAChR channel profile. We are aware that even massive surges in mRNA levels do not reliably predict changes in levels of encoded product. As such, the molecular analysis is done in the context of (often in the same cell as) detailed electrophysiological study of the nAChR channels.

→**Expt 1**: Are the levels and/ or profile of nAChR subunit mRNAs altered during the time of *in vivo* synaptogenesis? We will measure α2, α3, α4, α5, α7, α8, β2 and β4 mRNAs in VMN and MHN prior to, and following, contacting target SyMps or IPN, respectively. Tissue extracts of the VMN and MHN are prepared for first strand cDNA synthesis, and qPCR assays following methods as in [42]. Changes in the profile or level of nAChR mRNAs from the "pre-synaptogenesis" baseline are consistent with possible effects of input and/or target on nAChR expression.

Table A1-1: The profile of nAChR subunit gene expression in the visceral motor & medial habenula nuclei changes during *in vivo* synaptogensis.

	α3	α4	α5	α7	β2	β4
Visc. Motor						
E18 vs. P0 mouse	▲	▲	▲	▲		ND
E 9 vs. E18 chick	ND	▲▲	▲	▲		▼
Med. Habenula						
E16 vs. P0 mouse	▲	▲	▲		▲▲	ND
E 11 vs. E17 chick	▼		▲	▲▲	▲▲	▲▲

qPCR assay of chick tissue extracts; mouse data from "side-by-side" in situ assays ([FN 1], Methods). ND: not determined; - no change or low signal.

Preliminary studies reveal significant increases *and* decreases in the levels of specific nAChRs mRNAs in both VMN and MHN during development (**Table A1-1**). DiI injections and immunohistochemical studies indicate that chick MHN and VMN initially project to their targets by ~E9; in mouse cholinergic projections are detected within target IPN and in SyMps by E16.

→**Expt 2**: Are developmental changes in nAChR expression *in vivo* recapitulated by target-neuron contact *in vitro*? We will test whether contact with target SyMp or IPN alters nAChR subunit mRNAs in presynaptic VMN or MHN neurons *in vitro*. All cell cultures are prepared from "synaptically-naive" tissue (i.e. removed prior to the establishment of target interactions *in vivo*; see [42,43,64,215]**Progress** & Methods).

Following electrophysiological assay of nAChR-mediated currents (see below), the cytosol of individual neurons is collected and the sample processed for qPCR using our standard procedures and controls.[42]

Preliminary studies: The expression of CNS nAChRs is strongly regulated by target interactions. In fact, co-culture of chick VMN neurons with target SyMps qualitatively recapitulated the normal developmental changes in nAChR profile (**Fig. A1-1: Table A1-1**). The regulation of α3 and α5 in developing chick MHN *in vivo* did not match target contact associated changes seen *in vitro*. These initial results could be in error (n=2 for each assay), or α3

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Number pages consecutively at the bottom throughout the application. Do not use suffixes such as 3a, 3b.

and α5 may be regulated by non target-derived factors. <u>Preliminary studies</u> of developing mouse VMN and MHN show changes in nAChR subunit gene expression similar to those in chick: (**Fig. A1-1; Table A1-1**).

Aim 1 Study 2: <u>Ar neuronal responses to nicotinic agonists influenced by interactions with post-synaptic partners?</u> We will complement measures of nAChR subunit mRNA (as above) by the following physiological assays of nAChR protein complexes (*albeit* limited to surface protein and nAChR complexes that are gated by agonist). <u>Previous studies</u> of nAChR expression and nAChR mediated currents in chick PNS revealed coordinate changes in these parameters with innervation of non-neural target tissues (see[42,43,215] & **Progress**).

→ <u>Expt. 3:</u> <u>Macroscopic current responses to applied agonists and antagonists</u> will be recorded by whole cell patch clamp of VMN or MHN neurons removed prior to target interactions *in vivo* and maintained *in vitro* ± target SyMp or IPN neurons. Alterations in the number of surface nAChR channels is measured by comparing the magnitude of macroscopic currents elicited by maximal concentrations of agonist (I_o; corrected for cell capacitance). Alterations in the underlying nAChR subtypes might be evident as changes in agonist and antagonist pharmacology or as changes in the activation and desensitization kinetics of agonist-gated macroscopic currents, as we have previously detailed (e.g. see **Progress**). We will assay the concentration-dependence of the macroscopic current responses and desensitization profiles with ACh (5-7 doses between 1 – 500 μM) and nicotine (0.1-50 μM). We also will examine the susceptibility of agonist evoked currents to nBgTx (10 vs. 100 nM), αBgTx (10 vs. 500nM) and MLA (0.1 vs.10 nM). Experimental protocols for recording, analyses and drug application (by pressure ejection or local micro-perfusion with opposite-directed macro-perfusion) have been published[218,219]. In view of results presented in **Fig. A1-1**, we predict significant changes in ACh-elicited responses consistent with alterations in the number and subtypes of nAChR channels.

→ <u>Expt 4:</u> <u>More detailed analysis of changes in the profile of nAChRs within the somata-dendritic domains of VMN and MHN neurons will be assayed using single channel recording.</u> Single channel events elicited by low agonist concentrations (e.g. 5 μM ACh; 0.1 μM nicotine) are collected by continuous recording in cell attached patches. Records are analyzed for the presence of one or more nAChR subtypes based on distinct chord conductance (γ_c; from –90 to –40mV holding potential), open duration kinetics (τ_o) and probability of opening (Po) as a function of continuous exposure to agonist (i.e. probability density functions) as previously described.[18,123 Yu. 1998 #379,124,218]

<u>Preliminary recordings</u> of nAChR single channel currents in P0 mouse neurons reveal multiple channel subtypes, distinct in γ_c, τ_o and P_o (inset panel: calib: 1pA x 2ms). Current/voltage (I/V) plots indicate rectification of some subtypes at strongly hyperpolarized and/or near zero transmembrane potentials. All I/Vs are ohmic between – 90 and –40 mV, the range chosen for chord conductance measures. Prior single channel studies of chick SyMp AChRs ± non-neural targets, revealed target-specific changes in the nAChR channel expression profile ([42]& **Progress**).

Aim 1 Study 3: <u>Determine if the distribution of nAChRs between somata-dendritic vs. axonal d mains of CNS cholinoceptive neurons is altered by interactions with post-synaptic partners.</u> Initial studies, using Ca imaging and focal application of nicotine, suggest that the targeting and/or clustering of nAChRs at presynaptic terminals of VMN or MHN neurons requires direct contact with target neurons[69,115]. Thus, we can detect presynaptic nicotine-induced increases in Ca-signaling *only at the site of neurite-neurite contact* and, rarely, along axons within large fiber bundles. Although these findings may reflect the actual distribution of nAChRs (i.e. low along axons, high at sites of contact) the possibility remains that the assay is not sufficiently sensitive. We will determine the role of neuron-neuron interactions in targeting nAChRs to axonal domains in *in vitro* preparations using a variety of techniques chosen to improve resolution and reliability.

→ <u>Expt. 5:</u> <u>Electrophysiological assays of somata-dendritic nAChRs.</u> VMN or MHN neurons are removed prior to target contact *in vivo* and maintained *in vitro* as "micro-thinned" explants (Methods) in the presence or absence of target neurons. Neurite-neurite contacts will be visualized with Nomarski DIC optics. ACh (3mM + 1uM atropine) is applied from a fine tipped (<1μm) pressure ejection pipette to regions of the neuronal soma and processes to optimize focal agonist application (area ~10-15 um vs. >100 um by standard protocol). Whole cell voltage clamp recording. (+TTX) reveals the relative sensitivity of 5-10 distinct sites within ~100μm radius of the cell soma. The nAChR-mediated responses are analyzed as I_o corrected for length constant and distance from the soma. Preliminary results using this approach are shown in **Fig. A1-2 (top)**.

→ <u>Expt 6:</u> <u>Ca imaging assays of axon & axon terminal domain nAChRs.</u> Complimentary studies mapping the distribution of nAChRs along neurites and at presynaptic sites of VMN-SyMp and MHN-IPN contact utilize Ca-imaging of fura-2 AM treated neurons. As in previous studies, photometry and ratiometric measures of the time course and extent of changes in [Ca] are determined in response to focal nicotine application. We have achieved considerable improvement in our signal-to-noise by including Mn^{2+} in the intracellular recording solution to quench postsynaptic, nicotine-evoked changes in [Ca]. **Fig. A1-2 (bottom)**. The use of a cooled-CCD camera is required to optimize signal sufficient for reliable detection of presynaptic nAChRs on single axons and at presynaptic boutons.

Although E18.5 and P0 hybridizations were processed together, thus controlling for probe labeling, hybridization and wash conditions, and development time, this comparison is qualitative and, as such, requires the quantitative analyses proposed.

→ **Expt 7:** Changes in the nAChR distribution within the somata-dendritic domains of VMN and MHN neurons will be assayed in more detail by single channel recording. These studies employ the technique of "patch mapping" developed and utilized by us ([123] & Methods). This technique assays nAChR channels on an individual neuronal soma by repeat cell-attached patch recording at >5 sites. The *clustering* of nAChRs is evident in single channel records as patches with multiple channels (overlapping events) interspersed with patches that have no detectable openings. [18,123] *Segregation* of nAChR channel subtypes is evident as patches that include openings of only one nAChR subtype (distinguished by measurement of γ, average τ_o and P_o). Both "phenotypes" of nAChR maturation were detected with innervation of chick SyMps *in vivo* and *in vitro*[123,124]. Previous studies of acutely dispersed MHN neurons unveiled developmental changes in the profile of somatic nAChR channels during synaptogenesis *in vivo*. Comparison of typical single channel records from E11 vs. E17 chick MHN neurons reveals more "high density" nAChR patches (clustering?) and fewer mixed nAChR-subtype recordings (segregation?) at E17 ([18], enclosed).

→ **Expt 8:** Regulation of axonal targeting of nAChR channels assayed by measuring nicotine stimulated transmitter release. A simple and sensitive assay of nAChRs in axonal domains is to monitor nicotine induced presynaptic facilitation (as in [69,70,115]). In these experiments whole cell or perforated patch recording is used to record spontaneous (sPSC) and evoked postsynaptic currents (EPSC) before and after focal application of nicotine. Obviously, this approach is limited to studying presynaptic nAChRs in neurons following target contact.. Growing neurites and initial contacts are readily visualized in co-cultures examined within 24 hours of plating pre- with postsynaptic neurons. The onset and development of nAChR-mediated synaptic facilitation will be monitored by continuous recording in visually contacted, postsynaptic SyMp or IPN neurons. Measurement of sPSC frequency with nicotine application to sites of presynaptic-postsynaptic contact will be used to assess the time course and extent of nAChRs targeting to VMN or MHN presynaptic terminals.

Previous imaging and physiology studies document that nAChRs are targeted to presynaptic sites within ~48 hours of chick VMN-SyMp or MHN-IPN neuron co-culture [69,70,115]. Preliminary studies in TTX-treated IPN slice from P0 mouse, confirm the presynaptic localization of nAChRs at "real" MHN-IPN synapses. Application of 10 μM nicotine in the vicinity of v-clamped IPN neurons in the slice elicits a brisk increase in synaptic current frequency without changes in sPSC amplitude (sEPSCs. **Fig. A1-5, below**) that is sustained for ~5 min. consistent with presynaptic nAChRs on excitatory afferents. Although SyMp and IPN neurons are cholinoceptive, the apparent K_m of somata-dendritic nAChRs is several orders of magnitude higher than that of presynaptic nAChRs (see in [115], enclosed). We do not detect direct current responses in SyMp or IPN post-synaptic neurons.to the low doses of nicotine (≤ 1 μM) used in these studies.

→ **Expt 9:** Assay of target-contact induced changes in the distribution of ecto-domain tagged nAChRs. Although the laboratory has considerable experience with the above techniques, the Ca imaging and recording assays are limited in sensitivity and in spatial resolution, respectively. We have developed one more approach to assay nAChR distribution. These studies employ adenovirus-mediated infection of primary chick and mouse neurons with constructs encoding ecto-domain tagged nAChR subunits (see Methods). VMN or MHN neurons, removed prior to *in vivo* target contact, are infected with epitope-tagged versions of $\alpha 7$, or of $\alpha 4$ and $\beta 2 \pm \alpha 5$. The distribution of surface nAChRs is mapped by fluorescence imaging of epitope-immunoreactivity in living neurons maintained either alone or in the presence of target neurons. Preliminary studies confirm that FLAG-tagged nAChR subunits participate in functional nAChR channels and are detectable in both somata-dendritic and axonal domains of neurons *in vitro* (data not shown & see **Fig . A2-3**, below).

Aim 1. Part 2: Do cholinergic afferents regulate pre- and postsynaptic nAChRs in the CNS?

Our preliminary studies and published work document significant changes in the expression of specific nAChRs following the arrival of cholinergic projections in limbic telencephalic and diencephalic structures of chick, mouse, rat and humans.[16,84,121,138,174,223] In view of these findings, and the important role of cholinergic afferents in the regulation of nAChRs in the PNS, we propose the following experiments to test whether cholinergic input regulates nAChRs at two important sites of cholinergic projection in the CNS: the IPN and amygdala.

The decision to focus these studies of nAChR regulation in IPN and amygdala is based on previous studies in chick, rat and human and on results of our preliminary survey of the development of cholinoceptive synapses in mouse CNS. In sum, 1st: Both sites are heavily innervated by basal forebrain cholinergic nuclei and include neurons with robust nAChR expression. 2nd: Pre- and/or postsynaptic nAChRs have been implicated in controlling synaptic excitability within both these brain regions. 3rd: Functions subserved by both regions appear to be compromised in neurodegenerative diseases such as Alzheimer's and Parkinsons (see Background). In addition, 4th: Analysis of cholinoceptive sites in mouse IPN affords us the opportunity to compare results obtained in mammalian CNS with findings from studies of avian IPN. 5th: Analysis of synaptic development of mouse IPN *in vivo* constitutes the fundamental groundwork for **Aim 2** studies of the CRD-NRG-- mouse. On the other hand, we are aware of potential difficulties in studying synaptic maturation in mouse IPN, due to the early development of this region and the general view that it is difficult to get quality recordings in prenatal brain slices. In other preliminary studies, we found that 6th: The development of cholinergic projections to and maturation of cholinoceptive synapses within amygdala occur largely postnatally, and 7th: The regions of cholinoceptive synapses within the amygdala are well defined suggesting that developmental studies of cholinoceptive sites in mouse amygdala should be more technically straightforward. If

recording from embryonic IPN in slice preparations proves more problematic than our preliminary studies suggest (see below), we will modify the experimental plan as follows. First, electrophysiological studies of mouse IPN will utilize cell culture and synaptic co-culture preparations. Second, the proposed electrophysiological studies of nAChR maturation *in vivo* will be confined to studies of amygdala slice preparations.

Aim 1 Study 4: Determine if contact by afferent projections regulates the expression of nAChRs.

➜ **Expt 10:** We will determine the time course of cholinergic projection to the IPN and amygdala *in vivo*. Developing cholinergic neurons and their projections are mapped with protein and mRNA probes to the Vesicular Acetylcholine Transporter (VAT) and by a modified AChE histochemical method in fresh frozen or fixed/ frozen cryostat sections (Methods). Cholinergic projections to the IPN emanate from the vertical limb of the Diagonal band N. (DBv), the medial septal and the dorsolateral tegmental nucleus. Cholinergic afferents to the amygdala arise from the horizontal limb of the Diagonal band N. (DBh), the nucleus Basalis of Meynart, and the magnocellular preoptic area, via the medial forebrain bundle. Neither the IPN nor amygdala has intrinsic cholinergic neurons.

➜ **Expt 11:** The pattern of nAChR subunits expressed in pre- and postsynaptic neurons will be assessed by ISH, using ^{35}S-labeled, rather than digoxin-labled, probes to α3, α4, α5, α7, β2 and β4 . Probes will be hybridized to tissue sections (as above), and the extent of hybridization quantified using the slide scanner and autoradiographic image analysis system requested.

➜ **Expt 12:** We will further quantify *in vivo* developmental changes in nAChR subunit expression by qPCR assay of mRNAs from tissue "punches" of IPN and amygdala obtained before and after the arrival of cholin rgic projections. Preliminary data on mouse IPN and amygdala (summarized in **Table A1-2**) include the detection of cholinergic input as early as E16 in IPN, whereas only growth cone (gc) tipped fibers are detected in E18.5/ P0 amygdala. The levels of expression and the number of IPN neurons with positive signal for each nAChR subunit mRNA are increased by P0 in mouse and rat (**Table A1-2**; **Fig. A1-3** & [206]). Likewise, there is significant upregulation of nAChRs concurrent with the arrival of cholinergic projections to two of the major cholinoceptive subnuclei of the amygdala-- the basolateral amygdaloid nucleus (BLA) and the nucleus of the lateral olfactory tract (NLOT). Based on the timing of the initial nAChR induction within many CNS regions, we focussed our proposed mouse studies on the IPN (E16-18) and amygdala (P0-P7). In both regions, the pre and postsynaptic elements can be reliably identified and isolated throughout the period of likely input-dependent nAChR regulation.

TABLE A1-2	AChE Fibers	α3	α4	α5	α7	β2
IPN						
E16	+	-	-	+	-	++
E18/P0	+++	+/-	+	++	+/-	++
P7	+++	++	++	+++	++	+
AMYG						
E16	-	-	-	-	-	-
E18/P0	+ (gc)	+/-	++	+/-	+	+/++
P7	+++	ND	+++	+	+++	++

"Amygdala" refers to 2 major cholinoceptive subregions, the basolateral nucleus (BLA), and the Nucleus of the lateral olfactory tract nuclei (NLOT). (gc)= growth cone tipped AChE + fibers. ND= not determined

➜**Expt 13:** We will determine if cholinergic afferents regulate the nAChR subunit mRNA profiles in CNS neurons *in vitro*. The IPN can be removed from chick or mouse prior to innervation *in vivo* and maintained *in vitro* in the presence or absence of presynaptic MHN neurons, a prominent source of cholinergic and non-cholinergic afferents. We collect cytosol for single cell qPCR following electrophysiological characterization and assessment of the innervation status of the neuron studied (see below). Note that the protocols involved in these and in the following *in vitro* studies of IPN neurons ± MHN input are identical to those outlined in **Aim 1 Studies** 1-3. Although we present this study separately for intellectual clarity (I hope), the analysis will be conducted on the same *in vitro* preparations. Preliminary studies indicate that α3,4,5,7, β2 and β4 subunit genes are expressed by E10 chick IPN neurons and that *in vitro* innervation by MHN significantly up or down regulates the levels of all subunit mRNAs except α5 (**Fig. A1-3**).

➜ **Expt 14:** We will determine the impact of cholinergic afferents on nAChR expression in individual neurons of mouse IPN and amygdala, following electrophysiological characterization of intact (slice) preparations. Slice preparations including the region of the IPN and surrounding afferent fibers are prepared from E16, E18.5/P0 and P7 animals (Methods). Slice preparations of the amygdala are prepared so as to include the BLA and NLOT of P0, P7 and P14 mice (see Methods). Collection of individual neuronal cytosols and qPCR assays will be performed following characterization of the electrophysiological profile and marking of the neurons for subsequent relocation and AChE staining (as in Aim 1 Study 1, & see below).

Aim 1 Study 5: Determine if input regulates nAChR-mediated currents in CNS neurons.
Preliminary results of **Expts** 10-14 suggest that cholinergic projections arrive just prior to observed changes in nAChR subunit gene expression in mouse IPN and amygdala (~ E17-P0 and P0-P7, respectively). The timing of these events is, therefore, at least consistent with cholinergic afferents exerting an instructive role in the regulation of nAChR profile. This temporal correlation is not terribly imformative however, since synaptogenesis by extrinsic and intrinsic *non-cholinergic* neurons occurs during the same general time. We will determine the role of cholinergic and non-cholinergic afferents in controlling nAChR profile by manipulating the neuronal interactions of P0 amygdala in *in vitro* cultures and co-cultures. The amygdala is well suited to these experiments because of the absence of intrinsic cholinergic neurons and the fact that the BLA and NLOT are normally interconnected with adjacent subregions of

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Principal Investigator/Program Director (Last, first, middle): Role, L. W. NS29071.

amygdala. We measure changes in the number and profile of nAChR channels in cultures of BLA and NLOT alone with those in co-cultures of amygdala with septal cholinergic area microexplants (see Methods) before and after contact by cholinergic vs. non-cholinergic afferents.

→**Expt 15: *In vitro* studies:** Regulation of nAChR-mediated currents in IPN neurons by presynaptic input and cholinergic projections will be assessed in dispersed cultures of IPN with and without MHN. Innervation status is assessed by voltage clamp recording of spontaneous (sEPSCs) and evoked synaptic currents. We will determine the pharmacological and biophysical characteristics of nAChR channels by analyzing macroscopic and single channel currents **(Aim 1 Study 2)**. *In vitro* physiological analysis and collection of samples for qPCR is followed by recording of the x and y co-ordinates and acquisition of a video image. On completion of the experiment, cultures are fixed and cholinergic fibers visualized by immunohistochemical staining of AChE and VAT (see Methods). Recording and sample collection protocols for these *in vitro* experiments are identical to those above. Preliminary studies demonstrate that nAChR-mediated currents in IPN neurons are regulated by presynaptic input from MHN neurons in synaptic co-cultures. The amplitude of ACh-elicited macroscopic currents is greater in innervated IPN neurons co-cultured with MHN compared to "IPN alone" sibling cultures **(Fig. A1-3)**. *In situ* and immunohistochemical probes to cholinergic markers (VAT, ChAT, and AChE) confirm that IPN and amygdala are devoid of *intrinsic* cholinergic neurons and that explants of MHN include cholinergic **(Fig. A1-4)** and glutamatergic projection neurons.

→ **Expt 16: Slice studies:** Analysis of nAChR–mediated currents in intact (slice) preparations of mouse IPN and amygdala test for changes in nAChR physiology concurrent with innervation. These studies assay macroscopic (and if possible, single channel) currents in slice preparations of IPN and amygdala. Agonist-elicited macroscopic currents are recorded in neurons within the slice using perforated patch recording. I_o is measured, corrected for capacitance and plotted vs. agonist or antagonist concentration in studies testing nAChR pharmacology **(Study 2 above)**. The innervation status of each neuron is assessed by recording spontaneous synaptic activity (with and without TTX) and by bipolar electrode stimulation of the afferent fiber bundles surrounding the IPN. Evoked responses are elicited by stimulating within the lateral nucleus or by extracellular stimulation of the LOT, the major afferent sources to BLA and NLOT, respectively.

Preliminary studies demonstrate the feasibility of proposed electrophysiology studies in *in vitro* and slice recording preparations of IPN and amygdala **(Fig A1-5** and not shown). Initial recordings in slice preparations of mouse IPN reveal small macroscopic current responses and robust nicotine induced facilitation of synaptic currents **(Fig. A1-5)**. The ability to form high resistance seals (>5G) in the slice preparations bodes well for proposed studies of somata-dendritic nAChRs channels in intact IPN and amygdala.

Aim 1 Study 6: **Determine if afferent projections alter the distribution of nAChRs on individual CNS neurons.**

→ **Expt 17: Electrophysiological assays of nAChR distribution *in vitro*.** The distribution of surface nAChRs will be assayed in individual IPN and amygdala neurons removed prior to innervation *in vivo* and maintained *in vitro* ± explants of cholinergic afferents. We will map nAChR "hotspots" by focal application of ACh at the somata and multiple regions of the proximal neurites and axons as described.

→ **Expt 18:** Ca imaging assays of nAChR distribution *in vitro*. We will map nAChR distribution by Ca-imaging using the higher sensitivity cooled-CCD and the photometric and ratiometric methods described above. Analysis of changes in the clustering and/or segregation of specific nAChR channel subtypes within the somata-dendritic domains of IPN neurons will be assayed by "patch mapping" of IPN neurons.

→**Expt 19:** The distribution of nAChRs *in vitro* will be assayed following adenovirus-mediated transfer of epitope-tagged nAChR genes. We will extend preliminary studies of α4, β2 and α7 nAChR targeting to examine changes in receptor distribution associated with contact by cholinergic vs. non-cholinergic afferents. Low level illumination of immunofluorescent epitope-tagged nAChRs and physiological assays of nAChR responses will be used to examine nAChR distribution in living neurons before and after contact by presynaptic projections. Following *in vitro* assay of nAChR distribution, cultures are fixed, cholinergic fibers visualized by VAT immunoreactivity, and the pattern of VAT-positive - neurite contacts are compared with the distribution of epitope tagged nAChRs. Preliminary work demonstrates the feasibility of these approaches (see **Fig. A2-3, below**).

→ **Expt 20:** Assay nAChR distribution on CNS neurons in intact (slice) preparations. Studies of the distribution of nAChRs at CNS synapses in mouse will examine pre- and postsynaptic nAChR-mediated currents in individual neurons in slices of mouse IPN and amygdala. These experiments will examine neurons at more superficial locations within the slice to permit focal application of ACh and/or nicotine at the neuronal somata and proximal dendrites. Macroscopic current responses will be recorded and quantitated as described in **Aim 1 Study 3**. In all experiments the presence (or lack thereof) of synaptic input will be monitored electrophysiologically and contact by cholinergic afferents assessed by AChE histochemical staining of marked neurons (Methods; and see **Fig A1-5** and **Aim 1, Study 4**). The presence of presynaptic nAChRs on afferent projections within IPN and amygdala will be assayed by measuring the frequency of sPSCs in the presence and absence of nicotine. Nicotine is focally applied to the somata-dendritic region of the patch clamped IPN or amygdala neuron. Preliminary studies of mouse amygdala neurons in vitro suggest that nicotinic responses are rare before the arrival of (extrinsic) cholinergic

projections. However, with establishment of synaptic connections the number and extent of nAChR mediated synaptic facilitation is greatly increased.

AIM 1: SUMMARY, EXPECTED RESULTS AND POTENTIAL PITFALLS

Summary: Aim 1 studies test whether bi-directional communication among synaptic partners plays an essential role in the maturation of pre- and postsynaptic nAChRs. Proposed studies assess the contribution of neuron-neuron interactions in regulating the expression and cellular targeting of pre and postsynaptic nAChRs during development.

Expected Results: Although Aim 1 examines fundamental aspects of nAChR regulation in the developing CNS the proposed studies are in surprisingly uncharted territory. From prior analyses of PNS synapses, we expect that interactions with both pre and postsynaptic neuronal partners will influence the levels, profile and distribution of nAChRs. Although ambitious, our proposal to examine nAChR maturation in both intact and in co-culture preparations, will reveal the pattern of nAChR regulation by neuronal interactions in vivo and allow a more detailed, mechanistic analysis of these events, *in vitro*. Results from Aim 1 also constitute the essential "baseline" analysis of the maturation of CNS cholinoceptive sites. Aim 2 extends this work examining the role of specific signaling molecules and cascades controlling nAChR development.

Potential pitfalls: [1] An obvious gap in the proposal is the absence of studies using immunological or biochemical techniques to assay the expression and distribution of nAChR protein. Subunit specific probes exist, and they have been used in elegant immunoprecipitation and immunohistochemical studies of nAChRs (e.g.[6Torrao. 1997 #343,44,145,162]). The focus of this proposal is to compare changes in the nAChR subunit profile with alterations in the nAChR channel subtypes expressed by individual neurons. Clearly, the amount of nAChR protein in a single cell can not be quantified by protein or immunochemical techniques (even if I weren't biochemically-challenged). As such our assays of nAChR protein are necessarily confined to the functional, surface pool of nAChRs (i.e. as detected by electrophysiology). [2] The yield of Aim 1 studies largely depends on the success of the combined molecular and physiological analyses of embryonic chick and perinatal mouse CNS neurons in cultures and in slice preparations. Our experience with chick synaptic co-cultures is considerable: the feasibility of the mouse *in vitro* studies is documented in preliminary work. Although the slice techniques are undeniably new to the lab, we've begun, over the past 2 years to add mouse brain slice recording to our repertoire. In the context of collaboration with Dr. R. Axel on the physiology of mouse accessory olfactory bulb, N. Barazangi (key personnel) and Dr. Ron Yu (now in the Axel Lab) became proficient in slice recording of perinatal CNS tissue. I also updated my training by taking a "crash course" on preparing, and patch clamp recording in, mouse brain slices (a two-month "mini-sabbatical" in Dr. P. Ascher's laboratory). [3] In sum, preliminary studies demonstrate the feasibility of the proposed molecular and biophysical analyses. Prior work documents our success in similar studies of single cell qPCR and nAChR targeting in the PNS (see **Progress**). As we have already overcome the major technical hurdles of the proposed experiments, and several alternative approaches are delineated, the proposed studies have a high probability of generating substantial insight into the role of synaptic interactions in regulating CNS nAChRs.

AIM 2: IS CRD-NRG REQUIRED FOR SYNAPTOGENESIS-INDUCED CHANGES IN CNS nAChRs?

Overall Rationale for Aim 2 Studies:

Our previous work reveals that: 1: CRD-NRG, expressed by preganglionic neurons, is required for input-induced upregulation of postsynaptic nAChRs in chick sympathetic ganglia[215]. 2: CRD-NRG is expressed in brainstem visceral and somatic motor nuclei, and by midbrain and forebrain cholinergic neurons in chick, mouse and human.[207,209,215] 3: Most (if not all) cholinoceptive neurons in the CNS and PNS express NRG receptors (erbBs). The convergence of these data led us to hypothesize that CRD-NRG plays as important a role in the development and maturation of cholinoceptive synapses in the CNS, as it does in the PNS. Aim 2 studies test this hypothesis by assessing whether CRD-NRG/erbB mediated signaling controls the expression and targeting of pre and postsynaptic CNS nAChRs.

Overall Approach to Aim 2 Studies:

Proposed studies test the role of CRD-NRG in the regulation of nAChRs at identified sites of cholinergic projection and of nAChR, CRD-NRG and *erbB* receptor expression. We first test if CRD-NRG/erbB signaling mimics input or target-induced changes in nAChR expression *in vitro* by adding recombinant CRD-NRG or erbB receptor to "synaptically naive" neurons. **Studies 1-3** address the following questions: 1. Can activation of CRD-NRG/erbB signaling alter expression of nAChR subunits? 2. Does CRD-NRG/erbB signaling influence the number and profile of nAChR channels? 3. Does CRD-NRG/erbB signaling alter the targeting of nAChRs to somata-dendritic vs. axonal domains? Next:- the most critical tests of our hypothesis. **Studies 4 and 5** determine if CRD-NRG is required for input or target-induced maturation of nAChRs. *In vitro* analyses compare the expression, functional profile and distribution of nAChRs in synaptic co-cultures treated with control or antisense oligonucleotides targeted against CRD-NRG. Finally, we assess the role of CRD-NRG in the development of cholinoceptive synapses in studies of brain slices from WT vs. CRD-NRG⁻ mice. Studies of intact preparations will: 1st/ Extend preliminary anatomical studies establishing the pattern of expression of CRD-NRG and erbBs relative to synaptogenesis-induced changes in nAChRs and last (but not least), assess the impact of CRD-NRG on the maturation of nAChR subunit gene

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Principal Investigator/Program Director (Last, first, middle): Role: L.W. NS29071.

expression and targeting of nAChRs to cholinoceptive sites. The latter experiments compare WT, heterozygote CRD-NRG and CRD-NRG null mutant animals. Note that we have already generated the CRD-NRG-/- mice, completed the basic characterization of the "knock-out" phenotype and begun preliminary studies for this aspect of the proposal (see Progress).

Prior studies pertinent to Aim 2. Studies presented in the Progress Report demonstrate that the maturation of peripheral ganglionic and neuromuscular synapses *requires* CRD-isoforms of the Nrg-1 gene. Preliminary analyses of brain regions rich in cholinergic projections also implicate CRD-NRG in the maturation of cholinoceptive sites in the CNS. Table A2-1 presents some of these initial results and highlights data relevant to two predictions that stem from the hypothesis that CRD-NRG/erbB interactions regulate nAChR expression during synaptic development. First, if CRD-NRG is an important regulator of nAChR expression *in vivo* **then** one predicts that the normal developmental pattern of nAChR subunit gene expression should be disrupted in CRD-NRG null mice [CRD-/-]. Preliminary results support this prediction (highlighted blue). Second, one would predict that if CRD-NRG mediates the effects of synaptic partners in regulating the nAChR subunit profile, **then** added soluble CRD-NRG should mimic the effects of synaptic interactions *in vitro*. Preliminary assays are also consistent with this prediction (highlighted yellow). The data also hint at underlying complexity in that, in some cases, treatment with soluble rCRD-NRG recapitulates neither *in vivo* development nor *in vitro* synaptic interactions (e.g. red boxes). Nevertheless, as the most basic predictions are largely confirmed, CRD-NRG appears to be a worthy candidate for further study as a regulatory signal in the maturation of CNS cholinoceptive sites.

TABLE A2-1	α3	α4	α5	α7	β2	β4
VMNs In vivo (mice)						
DEV. Δ's: E16 vs. P0	↑	↑	-		↑	ND
CRD(-/-) vs. CONT.	ND	↓↓	-	↓·	↓	ND
(chick) _in vitro_						
Δ with target	↑	↑↑	↑	↑	ND	↑↑
Δ with CRD NRG	↓?	↑	no	↑	ND	↑↑
MHN In vivo						
DEV. Δ's	↑	↑	↑	↑	↑↑	↑↑
CRD(-/-) vs. CONT.	noΔ	↓?	↓	↓	↓?	ND
in vitro						
Δ with target	↑	↓	↓	↑↑	↑↑↑	↑
Δ with CRD NRG	↑	↑	↓	↑↑↑	↑↑↑	↑↑
IPN In vivo						
DEV. Δ's	↑↑	↑	↑↑	↓	↑	↑↑
CRD(-/-) vs. CONT.	*ND	↓	↓↓	no	no Δ	ND
in vitro						
Δ with input	↑	↑	↑?	↓	↑↑	↑↑
Δ with CRD NRG	↑	ND	↑?	↓↓	ND	~/?↑
AMYGD In vivo						
Δ DEV (P0 vs P7)	ND	↑	↑?	↑↑↑	n Δ	ND
Δ in CRD(-/-), P0 m.	ND	↓	↓?	↓?	no Δ	ND
E16 mouse _in vitro_						
Δ with input	~↑	~↑	~↑	↑↑	no Δ	ND

Mouse " in vivo" data refers to preliminary *in situ* analyses, All *in vitro* data refer to qPCR assays . no Δ : no change in subunit lev ls. ND= not determined ; ~ or ?: measurement uncertainty due to low "n" or low levels of expression. Also see Fig A2-2:

AIM 2: PROPOSED EXPERIMENTS

Aim 2 Study 1: _Determine if CRD-NRG/erbB- signaling regulates nAChR expression in CNS neurons._

The following experiments assess if changes in nAChR expression induced by neuron-neuron interactions are mimicked by treatment of "synaptically naive" neurons with rCRD-NRG or soluble erbB2 or erbB4 neuregulin receptors. If this first line of studies reveals that activation of CRD-NRG/erbB signaling *mimics* the effects of neuronal interactions, we will test if CRD-NRG is *required* for input or target-contact induced regulation of nAChR expression. The latter studies involve treatment of synaptic co-cultures with antisense oligonucleotides targeted against the CRD-NRG encoding mRNA to test if "functional deletion" of CRD-NRG disrupts neuron-neuron-induced changes in nAChR expression.

→**Expt 1:** Does treatment of CNS neurons *in vitro* with CRD-NRG alter nAChR expression? VMN, MHN or IPN neurons are removed prior to *in vivo* synaptogenesis and maintained *in vitro* in the absence of synaptic partners. Neurons are treated for 1-3 days with rCRD-NRG (from stably transfected HEK or COS cells; see Methods). As rCRD-NRG has yet to be purified (not that we haven't tried; see **Progress**), the semi-purified material is standardized as "activity units" based on levels of CRD-NRG-induced tyrosine phosphorylation relative to known quantities of bacterially produced, NRGβ1-EGF-peptide, or recombinant EGF (**Aim 3** and Methods). Sibling cultures are treated with media conditioned by cells transfected with an antisense CRD-NRG cDNA. Following treatment, neurons are collected for qPCR assay of α3.α4. α5, α7, β2 and β4 mRNAs (**Aim 1**, Methods). Preliminary studies comparing the effects of rCRD-NRG on chick MHN neurons with effects of contact with target IPN indicate similar (but not identical) changes in the profile of nAChR gene expression (**Table & Fig. A2-1**).

→ **Expt 2:** Does treatment with soluble erbBs or co-culture with erbB expressing cell lines alter nAChR expression in CNS neurons? We will test if endogenous NRGs expressed by VMN and MHN (but not IPN) neurons, mediate "back-signaling" following interactions with erbB receptors (normally expressed by target neurons).[50,65,92,135,155,207] Dispersed cultures of VMN or MHN neurons are treated with recombinant, soluble erbB2 and erbB4 (expressed as fusion proteins with Ig-Fc domain) for 1-3 days (see[59]; soluble erbB2 and erbB4 fusion proteins). Following treatment with soluble erbBs, the profile and level of nAChR subunit mRNAs expressed by VMN and MHN neurons is assayed by collection of the cytosol and qPCR (Methods).

Preliminary studies reveal that CRD-NRG/erbB interactions induce distinct intracellular signaling events in *both* the erbB expressing cell *and* the CRD-NRG expressing cell (i.e. NRGs appear to function as both ligand and receptor: see **Aim 3; Fig. A3-1**). Further credence to the concept of CRD-NRG "back signaling" is provided by

initial findings in both the PNS and CNS of the CRD-NRG⁻ mutant mice in which all neurons that normally express CRD-NRG are adversely affected (see [209], enclosed: Fig. 4B, panels K and I).

→ Expt 3: Does "functional deletion" of CRD-NRG disrupt synaptogenesis-induced expression of nAChRs in vitro Previous studies of VMN-SyMp synapses in vitro successfully utilized "poor-persons knock-out" strategies to demonstrate that CRD-NRG, expressed by chick VMN neurons, is required for the input-induced upregulation of nAChR expression in sympathetic neurons (**Progress**).[215] This technique, adapted for examination of nAChR channels in chick neurons under NS29071, involves the design of 15-18 mer oligonucleotide sequences complementary (i.e. antisense) to a specific region of a targeted mRNA. By combining sensitive bioassays with careful choice of the targeted sequence (and use of several control oligo's),antisense oligonucleotide techniques can dissect the functional contributions of proteins in developing neurons (e.g. see [18,101,115,150,215,217-219]). In particular, we used this approach to selectively delete the CRD-NRG or Ig-NRG isoforms from VMN neurons in VMN-SyMp co-cultures. Consequently, we were able to assess each of these NRGs contribution to the maturation of nAChR-mediated responses of innervated sympathetic neurons ([215] see Fig. 7). Proposed studies will utilize the same, well-characterized CRD-NRG targeted antisense and control oligonucleotides to test the requirement of CRD-NRG for input or target-induced changes in nAChR expression in chick CNS neurons. In these experiments "thinned" microexplants of VMN or MHN neurons are treated with 20 μM antisense (CRD-AS) or control/mismatch oligonucleotides (CRD-MM; added directly to modified culture media; see[18,101,115,150,215,217-219]). Oligonucleotides and media are refreshed daily for 2–3 days (sufficient for establishment of fiber outgrowth from the presynaptic explant) after which target neurons are added. Synaptic interactions and target-induced changes in nAChR expression are evident within 48 hours of co-culture. If CRD-NRG is required for the regulated expression of presynaptic nAChRs, then treatment of synaptic co-cultures with CRD-AS will disrupt the normal pattern of target-induced changes in the levels of the nAChR mRNAs. The nAChR mRNA profile in individual presynaptic VMN or MHN neurons will be measured by qPCR after electrophysiological characterization (below). Preliminary experiments indicate that CRD-NRG is required for the expression and/or targeting of presynaptic α7 containing nAChRs to VMN-SyMp synapses (see **Fig. A2-2**).

Aim 2 Study 2: Determine if CRD-NRG/erbB signaling regulates nAChR-mediated excitability of VMN or MHN neurons. The following experiments test if activation of CRD-NRG/erbB mediated signaling in "synaptically naive" VMN or MHN neurons mimics the effects of neuron-neuron interactions on the functional properties of nAChRs. If these experiments reveal that activation of CRD-NRG/erbB signaling mimics the effects of neuronal interactions on nAChR-mediated currents, we will test if CRD-NRG is required for regulation of nAChR-mediated excitability. These studies mirror those described in **Aim 2 Study 1** on CRD-NRG regulation of nAChR expression.

→ Expt 4: Can CRD-NRG/erbB interactions alter the profile of nAChR-mediated currents in CNS neurons in a manner similar to input or target-contact in in vitro preparations? The potential role of CRD-NRG/erbB interactions in the regulation of nAChR channels will be examined by assay of ACh-elicited whole cell and single channel currents in neurons treated with soluble rCRD-NRG or with soluble erbB fusion proteins. As in **Aim 2 Study 1**, this initial work tests if CRD-NRG/erbB interactions can mimic the regulatory effects of innervation and target contact on nicotinic responses. The cell culture and treatment protocols for these studies are identical to those described in **Aim 2 Study 1** and in fact, the experiments will be done on the same in vitro preparations. rCRD-NRG or soluble erbB will be added for 1-3 days to VMN, MHN and IPN cultures established from neurons prior to synaptogenesis in vivo and maintained in vitro without synaptic partners. The amplitude, agonist and antagonist pharmacology of nAChR – mediated macroscopic currents will be assayed using whole cell and perforated patch recording. If we find that activation of CRD-NRG/erbB interactions mimics the effects of input and/or target contact on nAChR macroscopic currents then single channel studies will be conducted to assess the profile of nAChR channel subtypes expressed. We predict that if CRD-NRG underlies the synaptogenesis-induced regulation of nAChR currents then treatment with rCRD-NRG or erbB fusion proteins, in the absence of synaptic interactions, would recapitulate the profile of nAChR channel subtypes expressed by input or target-contacted neurons.

→ Expt. 5: Is CRD-NRG required for synaptogenesis-induced regulation of nAChR-mediated currents? Preliminary work (**Aim 1**) points to the regulation of the profile of nAChR expression and the magnitude of nAChR-mediated macroscopic current responses in IPN neurons by MHN input. To test if CRD-NRG is required for input-induced changes in IPN nAChRs, synaptic co-cultures of MHN + IPN will be prepared and treated with CRD-AS or CRD-MM oligonucleotides. Following oligonucleotide treatment and the establishment of synaptic interactions, we will assay the biophysical and pharmacological profile of nAChR-mediated currents in innervated IPN neurons. Prior studies established that application of nicotine to either VMN-SyMp or MHN-IPN synapses evokes a robust facilitation of synaptic transmission due to the activation of presynaptic α7 containing nAChRs. The gating of these presynaptic nAChRs leads to increased Ca flux into the presynaptic terminal (detected by imaging of a Ca-sensitive dye; as described in **Aim 1** see **Fig A1-2**). Gating also increases the probability of release, as deduced from analyses of synaptic current frequency. Preliminary studies (**Aim 1 & Progress**) indicate that presynaptic nAChRs also are induced by target contact, but neither the molecular signal(s) nor the mechanism of induction is known.

→ Expt 6: To test if CRD-NRG is required for target neuron-induced changes in presynaptic nAChRs, synaptic co-cultures (i.e. VMN + SyMp or MHN + IPN) will be treated with CRD-AS or CRD-MM oligonucleotides. Following

treatment and the establishment of synaptic interactions. a detailed analysis of presynaptic nAChR-mediated nicotine-elicited synaptic facilitation will be undertaken. These studies involve focal application of nicotine at sites of VMN-SyMp or MHN-IPN contacts and recording of spontaneous (i.e. TTX resistant) and evoked synaptic currents. We predict that if CRD-NRG is required for the target contact-induced increase in presynaptic nAChRs, then treatment of synaptic co-cultures with CRD-AS should decrease nicotine-induced synaptic facilitation.

Preliminary studies utilizing this approach to assess the contribution of CRD-NRG to nicotine-induced synaptic facilitation in VMN-SyMp co-cultures are very encouraging (**Fig. . A2-2**). Whereas nicotine-induced facilitation is robust at all VMN-SyMp synapses tested under control conditions, CRD-AS treatment obliterates presynaptic facilitation. Note that CRD-AS treatment does not appear to disrupt synaptogenesis *per se* since both spontaneous and evoked synaptic currents are readily detected. This experiment also confirms previous studies implicating CRD-NRG in the regulated expression of postsynaptic nAChRs because the amplitude of the unitary synaptic current mode is decreased in CRD-AS treated cultures (see **Progress & Fig P1-1**). A potential problem arises with the analysis of presynaptic nAChRs in MHN-IPN co-cultures because only ~50% of the synapses are facilitated by nicotine under control conditions. Thus the proposed *in vitro* analysis of MHN-IPN synapses will require study of many synaptic pairs. Studies of MHN-IPN synapses in slice preparations from WT and CRD-NRG$^{-/-}$ mice will extend the cell culture analysis (see **Aim 2 Study 5**).

Aim 2 Study 3: __Determine if CRD-NRG/erbB-mediated signaling underlies input or target-contact induced changes in nAChR distribution in CNS neurons.__ Previous studies suggest that the clustering, subtype segregation and targeting of nAChRs to somata-dendritic vs. axonal domains are influenced by neuron-n uron interactions (see **Progress & Aim 1**). Thus, acute "patch map" recording of nAChRs in the somata-dendritic domains of mature SyMp or MHN neurons (E17: chick: [18,123]) reveals high-density clusters typically comprised of only one nAChR channel subtype. With maintenance of these neurons in vitro, the clusters "blur" - i.e. the nAChR distribution tends toward the diffuse pattern characteristic of synaptically naive neurons. If results from **Aim 1** support the idea that nAChR distribution is influenced by neuron-neuron interactions. we will pursue the following experiments.

→ **Expt 7:** Can CRD-NRG/erbB interactions alter the targeting of nAChRs to somata-dendritic vs. axonal domains? We will assess the potential role of CRD-NRG/erbB interactions in targeting of nAChRs in experiments where exogenous rCRD-NRG or erbB are added to isolated VMN, MHN or IPN neurons. Culture conditions and treatment paradigms are identical to those described above. Assays of the clustering, subtype segregation and targeting of nAChRs to somata-dendritic vs. axonal domains utilize protocols identical to those described in **Aim 1** (i.e. distribution of postsynaptic nAChR hotspots by focal ACh application and recording. repeat-patch mapping presynaptic nAChRs by measuring synaptic facilitation, and by mapping with Ca imaging, and overall nAChR distribution by visualization of epitope tagged nAChRs. In Preliminary studies we tested if rCRD-NRG alters the number and/or efficacy of nAChR targeting to presynaptic sites using 2 of the assays listed on both chick and mouse neurons. All studies indicated that rCRD-NRG regulates nAChR targeting. The effect of rCRD-NRG was tested in synaptic co-cultures of MHN + IPN and of mouse BLA amygdala + adjacent (lateral) amygdala (BLA afferents). Assay of nicotine-induced enhancement of sPSC frequency revealed significant increases in the extent of presynaptic facilitation in both types of synaptic co-cultures pretreated with rCRD-NRG. in addition. examination of epitope-tagged α4 containing nAChRs in MHNs previously subjected to Adenovirus mediated gene transfer), confirms that CRD-NRG increases the targeting of nAChRs to axonal and axon terminal domains (**Fig. A2-3**).

→ **Expt 8:** Is CRD-NRG required for synaptogenesis-induced changes in nAChR distribution? If the activation of CRD-NRG/erbB signaling alters nAChR distribution. we will test if CRD-NRG is required for establishing the mature pattern of nAChRs at CNS cholinoceptive synapses *in vitro*. We will determine if CRD-NRG is required for the clustering. subtype segregation or targeting of nAChRs to specific domains. Synaptic co-cultures of VMN-SyMp and MHN-IPN neurons will be treated with CRD-AS or -MM oligonucleotides for 2-3 days. as described in **Aim 2 Studies 1 & 2**. Assays of the effects of functional deletion of CRD-NRG on the distribution of nAChRs in somata-dendritic vs. axonal domains utilize protocols identical to those described in **Aim 1 Study 3**.

Aim 2 Study 4: __Determine if disruption of the CRD-NRG gene alters nAChR expression in mouse CNS.__ **Preliminary experiments** Table A2-1 compares the pattern of nAChR subunit expression in the VMN, MHN, IPN and amygdala of embryonic WT and CRD-NRG$^{-/-}$ mice. The results of this initial study indicate that genetic elimination of CRD-NRG disrupts the normal developmental pattern of nAChR expression. resulting in some seemingly dramatic differences in nAChR-mRNA signal in WT vs. CRD$^{-/-}$ mice (e.g. **Fig. . A2-4**). However. despite all precautions taken in the *in situ* hybridizations to avoid artifactual results. the technique used is neither quantitative nor sufficiently sensitive to support more than an *impression*.

→ **Expt 9:** Proposed studies assess the impact of CRD-NRG on nAChR expression in more detail. We will compare the spatial and temporal expression pattern of CRD-NRG and erbB2. 3 and 4 expressing cells in WT vs. CRD-NRG$^{-/-}$ mice at E14. E16. E18 and P0. These data define the normal pattern of CRD-NRG expression and assess whether CRD-NRG deletion has an impact on CRD-NRG expressing neurons. and/or erbB bearing cells. These studies focus on spinal cord and brain regions that include the cholinoceptive neurons characterized in **Aim 1** and prior **Aim 2** studies. i.e. the VMN (T1-L2. spinal cord). MHN. IPN and amygdala.

→ **Expt 10:** Subsequent studies extend initial work on the effect of targeted disruption of the CRD-NRG gene in the spatial and developmental pattern of cholinergic and cholinoceptive neurons (using probes to VAT mRNA and the full array of nAChR subunit genes) in selected brain and spinal cord regions. Possible disruption of CNS cholinergic projections in CRD-NRG-/- animals will be assessed in adjacent sections of E14, E16, E18 and P0 mice assayed with the VAT Ab and AChE histochemistry. Better quantification of nAChR subunit expression will be obtained by using radio-isotopic *in situ* hybridization probes and quantitative image analysis. The impact of CRD-NRG deletion on the absolute levels of nAChR subunit mRNAs will be assessed by qPCR analysis of nAChRs mRNAs in tissue punches of selected spinal cord and brain areas from E14, E16, E18 and P0 mice.

Aim 2 Study 5: **_Determine if targeted disruption of the CRD-NRG gene prevents the maturation of CNS cholinoceptive synapses:_** assay the functional profile and distribution of CNS nAChRs in CRD-NRG-/- mice. Aim 2 experiments proposed thus far are designed to assess if CRD-NRG/erbB signaling could regulate the development and maturation of pre and postsynaptic components of cholinoceptive synapses. This survey of potential effects of CRD-NRG/erbB signaling on nAChR expression *in vitro* is essential to understanding how this NRG isoform might participate in the developmental regulation of cholinoceptive synapses in vivo. Proposed studies of the effects of CRD-NRG deletion on cholinergic neurons and projections should provide insight into how the loss of CRD-NRG adversely affects the maintenance of central cholinoceptive synapses. In view of previous studies implicating CRD-NRG in the maturation of peripheral cholinoceptive synapses, we would predict (and preliminary studies confirm) that their initial formation in the CNS should proceed apace, even in animals deprived of CRD-NRG from the get-go. Proof that CRD-NRG is a required regulatory signal for the establishment of mature cholinoceptive synapses in the CNS, therefore, necessitates assay of nAChR-mediated responses after the initial period of synaptogenesis, in WT and CRD-NRG-/- mice.

→**Expt 11:** Maturation of cholinoceptive synapses in IPN of WT vs. CRD-NRG-/- mice. The MHN–IPN synapse provides the ideal locale for testing if CRD-NRG is necessary for the establishment of mature pre and postsynaptic cholinoceptive sites. Cholinergic projections to the IPN arrive early in mouse embryogenesis (see above). All of the cholinergic afferent nuclei (including a subset of neurons in MHN) express CRD-NRG; ErbB2 and 4 are prominently expressed in IPN by E16. In view of these data and the perinatal death of CRD-NRG-/- mice, proposed studies compare the cholinoceptive physiology of IPN neurons in WT vs. CRD-NRG-/- mice.

Preliminary studies indicate that slice preparations from E18.5/P0 animals are appropriate for these analyses. The actual developmental stage studied will be based on results of detailed **Aim 1** studies examining synaptogenesis and development of nAChR expression in the IPN. We will see if "knock-out" of CRD-NRG disrupts the maturation of presynaptic cholinoceptive sites by assaying nicotine-induced, synaptic facilitation in slice preparations of IPN. Slices are prepared for optimal preservation of the whorl of cholinergic and non-cholinergic afferents surrounding the IPN (as in **Aim 1**). Spontaneous synaptic currents are recorded in perforated patch configuration to maximize the duration of continuous recording. Local superfusion with low concentrations of nicotine activates presynaptic nAChRs, with minimal gating of the lower affinity, somata-dendritic receptors expressed by IPN neurons. Nicotine induced synaptic facilitation will be quantitated as the percentage increase in sPSC frequency compared with the pre-nicotine treatment event frequency (see: **Methods**). Facilitation of stimulus evoked release is measured by comparing the amplitude of evoked PSC's before and after nicotine treatment (**Methods**). An additional measure of presynaptic nAChRs is afforded by comparison of the number of failures in response to an applied stimulus of fixed intensity and duration. The pharmacological profile of presynaptic nAChRs, if present, will be assessed by quantitation of nicotine-induced synaptic facilitation in the presence and absence of available subtype specific antagonists (**Aim 1 Study 3, Methods**).

→**Expt 12:** The impact of the targeted disruption of the CRD-NRG gene on the maturation of CNS cholinoceptive sites will be studied by measuring the pharmacological and biophysical profile of somata-dendritic nAChRs in IPN neurons. These experiments will examine neurons at more superficial locations within the slice to permit focal application of ACh and/or nicotine at the neuronal somata and proximal dendrites. Macroscopic current responses will be recorded and quantitated (**Aim 1, Expt 3**) to assay for local nAChR "hotspots". The number of functional surface nAChR channels will be calculated from measurements of macroscopic currents elicited by maximal concentrations of agonist, corrected for cell capacitance. Single channel recording of somata-dendritic nAChRs, if feasible in slices, will allow a more in depth analysis of the γ and τo properties of the nAChR subtypes expressed. Note that clustering of nAChRs and maps of nAChR distribution can be assessed by low seal resistance "patch mapping" (1-5 G Ω) even if the high resistance seals required for collection of proper single channel records can not be attained.

AIM 2: SUMMARY, EXPECTED RESULTS AND POTENTIAL PITFALLS

Summary: Aim 2 studies test the hypothesis that CRD-NRG is a requisite regulatory signal in the establishment of mature cholinoceptive synapses in avian and mammalian CNS. Experiments comparing nAChR mediated responses of MHN and IPN neurons in normal mice and in mice genetically engineered to lack CRD-NRG isoforms, constitute the most strident test of the hypothesis.

Expected Results and potential pitfalls: Prior studies document the essential role of CRD-NRG/erbB signaling in the maturation of peripheral ganglionic and neuromuscular synapses. In view of these findings and other

preliminary work, we expect **Aim 2** studies to progress readily from the proposed tests of "can CRD-NRG mimic the effects of neuron-neuron interactions?" to the more interesting (and challenging) studies of if, where and how CRD-NRG signaling is required in the developing CNS. We expect that preliminary results for **Studies 1-4** will be confirmed, and that these studies will demonstrate that soluble rCRD-NRG mimics (a subset of) the regulatory effects of neuron-neuron interactions. Further, we expect they will show that CRD-NRG is required for expression and/or presynaptic targeting of nAChRs to (at least VMN) terminals. The outcome of analyses of the CRD-NRG-/- mice is more difficult to predict. Although preliminary results are encouraging, detailed studies of peripheral cholinergic synapses unearth more pleiotropic effects of disruption of the CRD-NRG gene, including an inability of cholinergic neurons to sustain synapses once formed (Wolpowitz et al., manuscript enclosed). Clearly, if CRD-NRG is required for the maintenance of CNS cholinergic projections and cholinoceptive sites, then the detailed anatomical and physiological studies proposed will unveil this novel and important role.

AIM 3: WHAT SIGNALING CASCADES DOES CRD-NRG/erbB ACTIVATE?

Overall Rationale for Aim 3 Studies:

Aim 3 initiates a mechanistic analysis of the signaling cascades underlying the regulatory activity of neuronal CRD-NRG/erbB interactions. The specific effects of CRD-NRGs on nAChRs and cholinoceptive synapses are one major theme of the studies in **Aims 1 & 2**. On a broader level, NRG signaling is implicated in various aspects of neural development and synaptogenesis in both the PNS and CNS[11,15,61,71,92,119,154,209,215]. Aim 3 studies are fundamental to understanding how this new family of neuroregulators exert their effects on neurons. First, we have strong preliminary data (to be confirmed and extended in **Aim 2**), that CRD-NRG/erbB interactions regulate the expression of nAChR subunit genes. This is likely to involve known signaling pathways downstream of erbBs in target cells that involve protein kinase cascades. Second, results of disrupting the exon that specifically encodes the CRD domain of the mouse Nrg-1 gene indicate that CRD-NRG isoforms are required for sustaining synapses between CRD-NRG expressing neurons and muscle or neuronal targets. In the periphery of mutants, the neurons that would normally express CRD-NRG, but not the erbB expressing target cells, die (see[209], manuscript enclosed). Centrally, it is clear that the neurons that would normally express CRD-NRG are adversely affected, but the details of these effects are unclear. The apparent lack of the maintenance of these neurons in CRD-NRG-/- mice is difficult to explain based on classic views of NRG/erbB signaling. This raises the possibility that CRD-NRGs function not only as ligand for erbBs, but as receptors for erbBs as well. Thus, the observed neuronal abnormalities must result either from a failure to establish a stable interaction between these neurons and target, and/or from a failure to elicit reverse or retrograde signaling via the membrane-tethered CRD-NRG.

Overall Approach to Aim 3 Studies:

Aim 3 Part 1 studies will identify which erbBs CRD-NRG can interact with and activate, and which of the downstream signaling cascades known to be activated by erbBs, but not NRGs. Results will be extended to analyze rCRD-NRG signaling in dispersed cultures of IPN and SyN neurons (erbB+/NRG-). Subsequently, we will compare the effects of rCRD-NRG on specific erbBs and downstream kinases, with the effects of co-culture with MHN or VMN, respectively. **Aim 3 Part 2** tests the hypothesis that the conserved cytoplasmic domains of CRD-NRGs are involved in retrograde signaling in input neurons, following establishment of interaction with target. Specifically we will determine: i/ if the NRG-1 cytoplasmic domain targets to the nucleus in an erbB-dependent fashion; ii/ if co-expression of the specific interacting protein, CNIP, alters this targeting; and iii/ as a long range goal, determine if the CRD-NRG-CYT has the ability to transactivate target genes.

AIM3 PROPOSED EXPERIMENTS

Aim 3 Part 1: Biochemical characterization of CRD-NRG signaling via erbB receptors. NRG-1 signaling to target cell nuclei involves sequential activation of erbB receptor tyrosine kinases, cytosolic serine/threonine protein kinases that translocate to the nucleus, and phosphorylation dependent activation of transcription factors (including both Ets and ATF/CREB family members)[10,36,37,55,61,62,86,91,113,133,134,144,175,179,186,187]. rCRD-NRG increases mRNA levels for nAChRs, including α3, α5, α7, β2 and β4 ([42,215] & see **Aim 2**). Little is known about the factors that regulate transcription of these genes in general, or that mediate the CRD-NRG response in particular.[12,20,35,43,63,82,166] In muscle, non-CRD-NRGs induce expression of nAChRs in a MAPK dependent manner that involves Ets family transcription factors.[3,4,26,61-63,166,184] Since the α7 and β4 nAChR gene promoters contain CRE-like and/or Ets-like response elements ([20,66]), we predict that CRD-NRG will activate the α7 and β4 genes, and probably the α3, α5 and β2 genes, in a similar fashion (see **Table A2-1**). To test this prediction, and to begin to clarify the role(s) of CRD-NRG both in regulating nAChR expression and in sustaining cholinoceptive synapses, we will define the pathways through which CRD-NRGs signal to neuronal nuclei.

→**Expt 1**: Determine the affinity and strength of CRD-NRG signaling through erbB receptors. We will confirm and extend preliminary studies demonstrating that the erbB2:4 dimer is the preferred CRD-NRG receptor (**Fig. P1-2B**). These studies utilize 32D hematopoietic cells expressing each of the known erbBs, singly or in pairwise

combinations ([190,198]; see letter from Dr. Wang, appended). ErbB expressing 32D cells will be used to determine the relative affinity and potency of signaling of CRD-NRG for each receptor type (EC$_{50}$s/ maximum mitogenic response).

→**Expt 2:** Demonstrate that CRD-NRG activates one or more MAPKs, and validate immunohistochemical methods for assaying MAPK activation in single cells. Demonstrating that CRD-NRG/erbB2:B4 interactions activate specific signaling kinases in target neurons requires that we be able to monitor these effects in small numbers, and in some cases, individual cells. This can be accomplished immunohistochemically using antibodies that specifically recognize protein kinases in their active state.[52,110,141] The putative transcriptional regulators of nAChRs include Ets and ATF/CREB family members. All three classes of vertebrate MAPK: the ERKs, JNKs and p38 kinases, phosphorylate one or more of these putative targets.[77,78,85,86,133,134,144,186] Therefore, we will assay CRD-NRG activation of ERK, JNK and p38 MAPKs. We have used phospho-MAPK antibodies to study EGF signaling in populations of cells, comparing MAPK activity measurements (i.e. μmoles PO$_4$ transferred/min/mg), with phospho-MAPK levels measured by immunoblotting.[24,185] To verify the immunological methods in single cells, we will use a cell line (T-47D) in which all known erbBs are expressed and functional.[10,55,113] T-47D cells treated with rCRD-NRG will be assayed for activation of erbB2 and erbB4 by measuring receptor phosphotyrosine content[215], and for activation of each of the three MAPKs. MAPK activation will be assessed by comparing results of direct kinase measurements[24,182,183,185]) with immunoblots of whole cell extracts probed with anti-phospho-MAPK antibodies (α-pMAPK)[110,140,141], and with whole cell and nuclear immunohistochemical staining with αp-MAPK Abs. We expect that CRD-NRG will activate ERKs, JNKs and possibly p38 kinases.[3,24,55,113,134,140,147,148,170,185,193] We anticipate that the α-pMAPK Abs are all they are marketed to be, and therefore we will show that CRD-NRG induces the rapid appearance of α-pMAPK staining, initially in the cytoplasm (within 1 - 5 min) followed by nuclear translocation (5 - 30 min) in virtually every treated cell.

→**Expt 3:** Demonstrate CRD-NRG activation of neuronal MAPKs. We will extend the above analyses with α-pMAPK Abs to synaptically naive IPN and SyMp neurons (**Aims 1 & 2**). Triplicate cultures (35 mm dishes) will be treated with semi-purified rCRD-NRG, control conditioned media, or rNRGβ1-EGF-peptide.[140,183,198] After ~30 min, kinase activation by each treatment will be analyzed by immunoblotting whole cell extracts. After 30 - 60 min (as, even in vitro, neuronal nuclei can 100u from receptors!), a set of dishes will be fixed and used for immunohistochemical staining. The third set will be cultured for 72 hrs, after which nAChR mRNAs will be measured by qPCR (**Aim 1 & Methods**). If, as we anticipate, CRD-NRG activates both neuronal MAPKs and nAChRs, we will use specific, cell permeable MAPK pathway inhibitors (e.g. PD98059 or SB203580: Calbiochem Inc.)[195] to determine if CRD-NRG activated MAPKs are <u>necessary</u> for CRD-NRG induced nAChR expression.

→**Expt 4:** Determine: (a) if input derived signals activate the same MAPKs as rCRD-NRG; and (b) if activation results from input derived CRD-NRG. Innervation of SyMp or IPN by CRD-NRG expressing VMN or MHN neurons respectively, induces nAChR expression ([42,215]: **Table P1-1; Fig. A1-3**). **Aim 2, Expt 5&6** are designed to determine if CRD-NRG is a (the) input derived signal that induces nAChR subunit expression. In this experiment we will use a similar approach to determine if input from VMN or MHN, activates the same panoply of signaling pathways as semi-purified CRD-NRG in SyMp or IPN. Synaptically naive SyMp and IPN neurons will be cultured in the presence or absence of input. Innervated and non-innervated cells (monitored electrophysiologically) will be fixed and stained with α-pMAPK. Additional SyMp and/or IPN neurons will be used for single cell qPCR measures of nAChR mRNAs. In this experiment, SyMp or IPN from WT mice will be: i/ cultured alone (no input); ii/ co-cultured with WT VMN or MHN (normal input expressing CRD-NRG), or iii/ co-cultured with VMN or MHN from CRD-NRG⁻ mice (normal input, but no CRD-NRG). We anticipate that innervation by WT MHN will induce nAChR expression and activate one or more, MAPKs (although probably not as robustly as acute stimulation with CRD-NRG). If this occurs, we will test the causal relationship between MAPK activation and nAChR induction, by including the cell permeable MAPK pathway specific inhibitors in the co-cultures. These inhibitors should prevent input from activating MAPK and inducing nAChR mRNA. →A long-range goal of these studies will be to extend the analysis of CRD-NRG signaling via MAPKs in vivo. We hope that by using the α-pMAPK antibodies, in conjunction with other techniques such as in situ hybridization for NRGs, erbBs and nAChRs, we will be able to compare normal synaptogenesis and aborted synaptogenesis in the CNS of WT, CRD-NRG⁺⁺ and CRD-NRG⁻⁻ mice (a continuation of studies in **Table A2-1**).[141,170,209]

Aim 3 Part 2: <u>**Determine if the cytoplasmic domain(s) of membrane tethered CRD-NRG mediates retrograde signaling following interaction with erbBs.**</u> Relevant preliminary results. CRD-NRGs are synthesized as transmembrane proteins with one of 3 cytoplasmic domains (referred to as CYT-a, b or c). The high degree of sequence conservation of these domains between species argues that CYT has an important function.[19,102,103,106,108,197] Four lines of evidence support the prediction that NRG-CYT functions as a retrograde signal. (1) In CRD-NRG⁻ mice, both peripheral and central neurons that normally express CRD-NRG, but not their erbB expressing targets, are adversely affected ([209], submitted manuscript enclosed). (2) When HEK293T cells were transiently transfected with either a NRG-CYTc-green fluorescent protein (GFP) chimera, or a similar chimera lacking the extracellular domains of NRG (TM-cyt-GFP), green fluorescence appeared at the plasma membrane and diffusely in the cytoplasm. The fluorescent signal translocated to the nucleus following either activation of PKC (with phorbol ester) or co-culture with erbB expressing cells (**Fig. A3-1**). (3) In dispersed SyMp cultures, a NRG-CYT

domain specific antibody (C-20; Santa Cruz) stained a subset of SyMps nuclei. (4) Using a yeast, two-hybrid screen, we identified a protein that specifically interacts with the NRG-CYT domain. This protein, CNIP (cytoplasmic neuregulin interacting protein), is novel (and therefore distinct from LIMK 1 which also interacts with the NRG-CYT; [197]), contains four putative zinc fingers, and is co-expressed with CRD-NRG in the developing CNS. Because of these observations, we hypothesize that membrane tethered CRD-NRG functions, not only as a ligand for erbBs on target cells, but also as a receptor for erbBs. When bound by erbB "ligands", CRD-NRG undergoes proteolysis and the CYT domain elicits retrograde signaling responses, following nuclear translocation. We will extend preliminary studies to test this hypothesis.

→**Expt 5:** Determine if interaction between membrane tethered CRD-NRGs and erbBs stimulates nuclear translocation of NRG-CYT. CRD-NRG-CYTa-GFP chimeras will be transiently expressed +/- CNIP, in HEK cells. Transfected cells will be cultured alone (negative control), co-cultured with erbB expressing cells (T-47D cells), treated with soluble erbB4 (CRD-NRG binding) or erbB2 (non-CRD-NRG binding) receptors[59,83], or with PKC activators (positive control). Live cells will be monitored for nuclear GFP. If a significant percent of CRD-NRG-CYTa-GFP enters the nucleus following stimulation, cell extracts and conditioned media will be immunoblotted with antibodies specific for extracellular or intracellular NRG epitopes. Release of soluble CRD-NRG will be assayed in receptor tyrosine phosphorylation assays[215]. This experiment will demonstrate if the translocation of NRG-CYT to the nucleus is accompanied by proteolytic processing and release of soluble CRD-NRG, and if co-expression of CNIP influences cleavage and/or nuclear translocation. Based on preliminary results (see also [197]) we predict that receptor interaction (erbB4 soluble and surface associated) and PKC activation will stimulate CRD-NRG proteolysis and nuclear translocation of the CYT domain.

b→**Expt 6:** Determine if erbB4 interaction stimulates processing and nuclear translocation of neuronally expressed CRD-NRG-CYT. CRD-NRG expressing neurons (VMN, MHN) will be (a) cultured alone, (b) treated with soluble erbB4, erbB2 or PKC activators, or (c) co-cultured with T-47D cells. At time points spanning the optimal times for stimulated translocation (**Expt 5**), cultures will be fixed and NRG-CYT localized by indirect IF, using the C-20 Ab. We expect that these results will mirror those of **Expt 5**, demonstrating that interaction of tethered CRD-NRG with erbB4 (soluble and membrane bound) will result in cleavage and retrograde transport of NRG-CYT to the nucleus. If this happens, we will extend these studies to co-cultures of IPN (erb B4+) and MHN (CRD-NRG+) neurons.

→**Expt 7:** Determine if NRG-CYT can transactivate a reporter gene. If, as we predict based on preliminary findings **(Fig A3-1)**, NRG-CYT inducibly enters the nucleus, we will determine if, once there, it is can affect transcription of a target gene.[176] Plasmids expressing either a fusion protein containing CRD-NRG-CYT and the Gal4 DNA binding domain (Gal4-$_{DBD}$), or CRD-NRG-CYT-Gal4-$_{DBD}$/VP16$_{AD}$ (the potent Herpesvirus VP16 transactivation domain) will be co-transfected with a Gal4-CAT reporter construct, +/- a CNIP expression vector, into HEK293T cells. Cells will be stimulated with erbB4 (soluble or by co-culture with T-47D cells) or PKC activators for 24-48 hrs after which CAT activity will be measured in cell lysates. Neither Gal4$_{DBD}$, nor VP16$_{AD}$ can efficiently enter the nucleus. Therefore, if the fusion protein(s) stimulate CAT expression, it indicates that the CRD-NRG-CYT has a dominant acting nuclear localization sequence that can "carried" the Gal4-$_{DBD}$ into the nucleus. In addition, if the fusion lacking the VP16$_{AD}$ is able to transactivate the reporter construct, NRG-CYT can function as a transcriptional transactivation domain. Transactivation of the Gal4-CAT reporter by NRG-CYT-Gal4-$_{DBD}$ would open the door for identifying target genes activated by retrograde NRG signaling in the CNS.

AIM 3: Expected Results & Potential Pitfalls.

The studies proposed in Expts. 1- 3 have a very high probability of success. All assays have been established in the 32D and T-47D cell systems except immunohistochemical staining (although we have extensive general experience with immunohistochemical techniques). In preliminary trials, we have demonstrated that single 35 mm dishes of primary cultured neurons yield sufficient protein for ready detection of total MAPKs by immunoblotting. Since, NRG typically activates 50 - 100% of the total MAPK, we do not anticipate technical trouble with these experiments. The details, strengths and drawbacks of neuronal culturing, qPCR and electrophysiological measurements of synapse formation are discussed in **Aims 1 & 2**. A potential complication to Expts. 4 will arise if electrophysiological measurements perturb basal activity of the various MAPKs. This will be determined in pilot experiments. If this occurs, we will determine the time necessary for MAPK activity to return to basal levels, prior to harvesting the treated cells for comparative assays. A second possible complication is that treating neurons with MAPK inhibitors alters their membrane potential. This too, is easy to determine in pilot experiments. If this is the case, inhibitor experiments in the co-cultures will be dropped. **Part 2:** We do not anticipate technical difficulty performing these experiments. We have obtained (and present) preliminary data for each of the experiments with the exception of treatment of cells with soluble receptors. The expression and purification system for the soluble erbB2 and erbB4 is currently being optimized and should be in place well in advance of the initiation of these experiments.

EXPERIMENTAL METHODS
Primary Neuronal cell culture, synaptic co-cultures and cell lines

MHN-IPN co-cultures: Embryonic chick MHN-IPN co-cultures will be prepared.[115,116] MHN thin sliced into explants from E10 chick or E 16 mouse will be plated on laminin-treated polyornithine-coated dishes. Maintenance overnight in minimal volume of media casues these microexplants to settle and flatten resulting in 1-3 cell thick , well adhered and physiology-friendly sections The next day dispersed IPN neurons from same stage embryos are added for co-cultures. Mouse MHN-IPN co-cultures are prepared and plated in similar conditions although a more acidic CO2 atmosphere than used with chick cultures appears to optimal.

VMN-SyN co-culture: E9 chick sympathetic neuronal cultures will be prepared.[157] Lumbar sympathetic ganglion chains will be dissected, neurons dispersed after brief trypsin treatment (0.01μg/ml. 15 min.) and then plated on PORN-coated culture dishes. Dorsal spinal cord from the mid-thoracic to the upper lumbar region (containing VMNs[143]), will be removed from E8 embryos. thin-sliced into microexplants. and added to SyNs that have been cultured for 2 days. After 14-18 hr, or overnight incubation for micro thinned explants additional medium is added. Cultures will be maintained for 2-3 days before being used for electrophysiological recordings.

Septal cholinergic -amygdala co-cultures: Embryonic mouse –amygdala cultures and co-cultures with septal cholinergic explants will be prepared using septal area explants from E16 mouse embryos. Microexplants are plated on laminin-treated. polyornithine-coated culture dishes. After 2 days, the BLA and NLOT region of amygdala dissected from E16 to P0 mouse embryos will be dispersed using papain-based protocol similar to [18] and plated either alone or with septal microexplants at a density of $5x10^4$ cells/dish.

Stable cells lines: Established cell lines. including MCF-7, T47D breast cancer cells. COS-7. HEK293T cells. and HEK293T derivatives stably expressing CRD-NRG (and anti-sense controls), will be maintained in DMEM + 10% FBS at 37° C.[215] Cells will be passed as necessary by trypsinization and replating. 32D cells. both parental and erbB expressing clones, will be cultured in DMEM supplemented with 10% FBS, IL-3 (present in conditioned media from WeHi cells) and NRGβ1-EGF peptide[140,198]

Anatomical Methods.

In situ hybridization: the distribution of mRNAs in brain sections will be assayed by in situ hybridization (ISH). Embryos will be perfused with 4% paraformaldehyde, post-fixed overnight at 4°C, cryopreserved in 30% sucrose and embedded in OCT. ISH [196] to target mRNAs will utilize riboprobes either synthesized using digoxin-conjugated NTPs. or for quantitative analysis. ^{35}S-αNTPs. Following hybridization and washing, digoxin labeled probe will be detected immunochemically using HRP-conjugated. anti-digoxin Abs. Radiolabeled probes will be detected by dipping slides in photographic emulsion (NBT/Kodak). After development. silver grains will be quantified using the Biologics image capture and analysis system.

Histochemical and Immunohistochemical Analysis: The distribution of proteins in cultured neurons and brain sections will be assayed using immunofluorescence. immunoperoxidase. and AChE histochemistry.[207-209,215,216] Cultures and tissue sections will be fixed in 4% paraformaldehyde/4% sucrose in PBS. and permeabilized with 0.25% Triton X-100 in PBS. Samples will be incubated with blocking serum. and then with optimal dilutions of primary antibody 2 hr-o/n). After PBS washes. sections will be incubated in the appropriate fluorescent or peroxidase-conjugated secondary antibody for 1 hour at 37°C. Staining (VECTOR VIP kit) and mounting (VectaShield. Vector Labs) will follow peroxidase antibody incubations. AChE staining will be performed as described.[180] The following antibodies will be used: goat anti-VAChT antibody (Chemicon). rabbit anti-FLAG antibody (Santa Cruz). monoclonal anti-MAP2 antibody (Chemicon). anti-phospho-MAPKs and anti-MAPKs (New England Biolabs). anti-NRG C-20 (Santa Cruz).

Physiological Methods:

Macroscopic and single channel recording: Tight-seal. whole-cell recording and single-channel recording will employ the patch clamp technique.[79] Amphotericin-permeabilized patches will be used in recording from IPN.[199] ACh will be applied by local pressure application within 10 μM of the soma. Whole-cell recording of spontaneous EPSCs in innervated neurons will be carried out in the presence of 2uM TTX to block action potential propagation. Sampling and analysis of individual sEPSCs will be carried out using analysis software written in Axobasic (as modified by R. Gird). Single channel recordings will employ the cell-attached configuration with pipettes containing 2.5 uM ACh.

Electrophysiological Analysis in Intact brain slice preparations: Mouse brains will be dissected into cold artificial cerebro-spinal fluid (mACSF) continuously bubbled with 5% CO_2/95% O_2. Brains will be sliced (400 μm sections) with a Vibrotome 1000. For amygdala slices. brains will be mounted coronally. Amygdala will be identified utilizing the appearance of hippocampal formation and the prominent fiber tracts from the internal capsule. For IPN slices. neocortical structures will be removed and the brain mounted coronally at ~30° angle toward the transverse plane so that the axonal connections between MHN and the IPN are preserved. IPN will be identified as the nucleus opposing the aqueduct. above the pons. surrounded by the peduncles in the tegmental area. Brain slices will be allowed to recover for 30 min (RT) in mASCF. continuously bubbled with 5% CO_2/95% O_2. Slices will be placed in

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Number pages consecutively at the bottom throughout the application. Do not use suffixes such as 3a. 3b.

the Olympus BX50WI infrared DIC microscope recording chamber. The chamber will be continuously superfused with mACSF bubbled with 5% CO_2/95% O_2. Patch pipettes will be guided to individual neurons by infrared visualization under higher power (40x). 4-8 MΩ patch pipettes will be filled with intracellular solution containing: 130 mM K^+-gluconate/5 mM NaCl/1 mM $MgCl_2$/.2 mM EGTA/10 mM Hepes/5 mM ATP/.3 mM GTP.

Calcium imaging of neuronal cultures and synaptic co-cultures: Fura-2 imaging of internal calcium ($[Ca^{2+}]_i$) will utilize a Zeiss IM 135-TV microscope equipped with a thermoelectric cooled-CCD camera (MicroMax 130-5Mhz-1300YHS, Princeton Instruments) or with an intensified CCD camera (Hamamatsu) and a Videoprobe imaging system (ETM Systems). Cells will be treated with fura 2-AM (10 uM) for 0.5-1 hr and washed before imaging. Ratios of fluorescent light intensity will be calculated and converted to $[Ca^{2+}]$. To map nAChR distribution in nerve fibers with calcium imaging, combined electrophysiological and fluorescence recording will be performed. Cells loaded with fura-2 AM will be mounted on the recording stage and an innervated IPN cell will be accessed for recording. In these experiments, the whole cell configuration rather than perforated patch will be used, and Mn^{2+} (0.5 to 5 mM) will be added to quench fluorescence from the soma.

Molecular Biological Methods:

Single cell qPCR assays: Cytoplasm from single neurons will be removed by applying negative pressure to a patch electrode after establishment of the whole-cell configuration. RNA will be reverse transcribed directly in 30 µl of 300 U RT, 40 U RNasin, 20µM random hexamers, 0.5uM of each dNTP for 1hr at 37°C. First strand cDNA will be amplified in 50 µl reactions (0.5 U Taq DNA Polymerase, 1mM primers, 200 µM dNTPs, including [α-^{32}P] dATP, 1.5mM $MgCl_2$) by 35 cycles of PCR. Ten µl will be amplified for 35 additional cycles in fresh reactions. Cycling conditions: 94°C, 30 sec; 45°C, 30 sec; 72°C, 45 sec. Products will be separated by electrophoresis and ethidium stained DNA bands will be cut out and radioactivity quantified by scintillation counting.

qPCR primer sequences The primer sequences used for amplification of chick nAChR subunit encoding genes (Ascension number, nucleotides of sense (S) and antisense (AS) primers): **actin**(L08165): S[860 - 877]/**AS**[1119 - 1135]; α2: S[1110 - 1127]/AS[1436 - 1453][3]; α3: S[897 - 914]/AS[1373 - 1390][128]; α4: S[1165 - 1182]/AS[1801 - 1818]; α5(J05642): S[480 - 500]/AS[1063 - 1083]; α7(X52295): S[974 - 991]/AS[1461 - 1478]; **α8:** S[931 - 955]/AS[1357 - 1380]; β2:S[785 - 802]/AS[1272 - 1288];[38] β4(J05643): S[745 - 764]/AS[1237 - 1256]. Mouse nAChR mRNAs will be amplified with sequences given in Drescher et al. (1995)[46]. *In situ probes:* CRD-NRG (AF045654, 607-1206), PAN-NRG-1 (AF045654, 867-1277), erbB3 (U29339, 3321-4113), erbB4 (L07868, 3088-3957), VAT (AF019045, 945-2157).

Packaging of Constructs in Adenovirus: Mouse and chick α3, α4, α5, α7, and β2 nAChR subunits N-terminally tagged with FLAG, HA, or Myc epitopes, will be "packaged" into recombinant adenovirus, which are able to infect post-mitotic cells.[80] nAChR cDNAs will be subcloned into pAdLox, a subgenomic adenovirus vector lacking the essential E1 gene. Co-transfection with a replication deficient adenovirus, ψ5, into CRE8 cells, followed by homologous recombination between viral sequences, results in production of virions containing pAdLox/nAChR sequences.

Characterization of CRD-NRG Signaling: CRD-NRG production: rCRD-NRG will be semi-purified from media conditioned by HEK293 cells stably expressing His6-tagged, chick CRD-NRGβ1a.[215] Media, conditioned for 48 h will be concentrated (Centriprep columns, Amicon), and used directly or further purified by Ni-affinity chromatography (Progress). Control media conditioned by HEK293 cells stably transfected with a plasmid encoding an anti-sense CRD-NRG will be processed in parallel. Recombinant NRGβ1 EGF-peptide will be purified following expression as a GST fusion protein in E. coli (provided by Dr. Wang, NCI).[140] Quantities of CRD-NRG in different preparations will be normalized by comparing induction of phosphotyrosine levels in MCF-7 cells relative to those induced by known quantities of the NRGβ1-EGF peptide.[215]

CRD-NRG/erbB interactions: Proliferation assays. 32D cells will be washed in PBS and resuspended in complete media, serum/IL-3 free media, or serum/IL-3 media supplemented with either rCRD-NRG or NRGβ1-EGF peptide.[129] ^{3}H-thymidine will be added from 44 - 48 hr later. Incorporation will be measured using an automated cell harvester.[140,183] Autophosphorylation assays. T-47D cells will be serum- starved for 24 hr and then treated with rCRD-NRG, NRGβ1-EGF-peptide or EGF (1-30 min). Protein lysates will be prepared in RIPA lysis. Tyrosine phosphorylated proteins will be immunoprecipitated with anti-phosphotyrosine antibody (mAb 4G10, UBI, Inc.). Immunoprecipitates will be separated by SDS-PAGE and blotted to nitrocellulose membranes. Filters will be probed with specific antibodies against ErbB1-4, and immunecomplexes visualized by ECL.[95] MAPK kinase assays: Immunecomplex kinase reactions. T-47D cells or primary neuronal cultures will be serum starved overnight, then stimulated with rCRD-NRG, NRGβ1-EGF peptide or EGF for 0-240 min. Lysates will be prepared and MAPKs immunoprecipitated from 200 ug extract protein. Immunecomplexes will be incubated with ^{32}P-γ-ATP and 2 µg substrate (myelin basic protein for ERKs, GST-Jun for JNK and GST-ATF2 for p38) at 30°C for 20 min. Reactions will be stopped by addition of SDS-PAGE sample buffer, boiled and products resolved by SDS-PAGE. Incorporation will be quantified either by scintillation counting of excised bands containing the substrates, or by phosphorimager.[24] Immunoblotting with α-pMAPK Abs. NRG activation of MAPKs will be assayed by probing immunoblots of cell lysates with antibodies that recognize the activated forms of MAPK. Primary antibody incubations will be overnight at 4 C, filters will then be washed, and incubated for 45 min with 2° Abs. After additional washes,

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immunecomplexes will be visualized by ECL. After recording the image on x-ray film. blots will be stripped and reprobed with the corresponding antibodies that recognize total MAPKs. Films will be scanned and the degree of activation calculated by the ratio of the α-pMAPK signal/α-total MAPK signal.[185] **Cell staining with α-pMAPK Abs**. Cultured cells will be fixed as described above and levels and subcellular localization of active MAPK visualized by immunohistochemistry.[110,170]

Nuclear targeting of NRG-Cyt: HEK293 cells will be transfected with plasmids encoding CRD-NRG-Cyt-GFP chimera (+/- a CNIP expression plasmid) using lipofectamine (GIBCO). After transfections. cells will be treated with phorbol esters. soluble erbB2 or erbB4. or co-cultured with T-47D cells. Cultures will be monitored by fluorescence microscopy for changes in the sub-cellular distribution of the GFP containing chimeric protein. In transient gene activation studies. HEK293 cells will be co-transfected with a gal4 UAS-CAT reporter plasmid. and expression plasmids encoding CRD-NRG-Cyt-Gal4$_{DBD}$ (DNA binding domain of the Gal4 protein) or CRD-NRG-Cyt-Gal4$_{DBD}$/VP16$_{TD}$ (Gal4 DNA binding domain and the Herpes virus VP16 transactivation domain) chimeras [176]. Cells will be treated as above. and 48-72 hrs later CAT activity will be measured. **Source of erbB2- and erbB4-Ig fusion proteins:** Soluble. erbB-Ig fusion proteins will be purified from conditioned media of transfected HEK293 cells by protein A-agarose affinity chromatography (the fusion proteins contain the Ig Fc domain.[39])

E. HUMAN SUBJECTS.

NONE

F. VERTEBRATE ANIMALS.

1. Mice. from either 129/Sv or C57Bl/6 inbred strains will be used in these studies. With the exception of stud males for breeding, all animals used will be females. embryos and pups within the first 10 days of life. We will attempt to generate the necessary mice from our own breeding colonies. but also anticipate that timed pregnant females will be purchased periodically to ensure an adequate supply of embryos at specific ages. We anticipate maintaing up to 30 cages for both the breeding colony and the housing of experimental animals. with less 5 mice/cage (or single nursing litters).

2. Mice have been chosen for these experiments because of the need to perform genetic manipulations that will alter specific aspects of nervous system development. Mice are the only species that offer the ability to both perform genetic manipulations and perform in vitro and in vivo developmental neurobiological and electrophysiological studies. Studies on these animals will complement studies done in chick embryos.

3. All animal handling and ultimate euthanasia will be done in the satellite animal facility on the 10th floor of the Psychiatric Institute Annex. All procedures will be performed by qualified animal technicians or individual investigators who have received specific training (lecture and wet labs) in animal handling offered by the staff of the Columbia University's Health Sciences Center Animal Care facility. All procedures will be done under the guidance of an attending Veterinarian.

4. The only manipulations that will be done on live animals will be breeding (natural—without exogenous hormonal stimulation of ovulation). and clipping of the ends of the tails in order to isolate DNA for PCR based genotyping. Clipping of tails will be done antiseptically by an AALAS certified animal technologist under veterinary supervision (by an institutional veterinarian) These procedures should cause little more than momentary pain or discomfort. Mice to be used for cell or tissue isolation will be euthanised by an overdose of carbon dioxide.

5. At the end of the protocols. animals will be euthanised by an overdose of carbon dioxide. This method follows guidelines established by the American Veterinary Medical Association Panel on Euthanasia.

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H. CONSORTIUMS/CONTRACTUAL ARRANGEMENTS.

NONE

I. CONSULTANTS.

NONE

REQUIREMENT FOR CYSTEINE-RICH DOMAIN ISOFORMS OF THE *NEUREGULIN-1* GENE IN SYNAPSE FORMATION

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Example 6:

Neuregulins constitute a large family of structurally related glycoproteins produced by alternative splicing of the *neuregulin-1* gene.[1,2] Neuregulin signaling, mediated by activation of the erbB family of receptor tyrosine kinases, has been implicated in the inductive interactions between pre- and post-synaptic partners at developing nerve-muscle and nerve-nerve synapses.[1,3-5] Gene targeting to selectively disrupt cysteine rich domain (CRD) containing NRG-1 isoforms in mice reveals that CRD-NRG-1 is required for synaptogenesis in the peripheral nervous system. In CRD-NRG-1 [(-/-)] mice, peripheral projections defasciculate and display aberrant branching patterns upon arrival within their targets. The profuse sprouts of motor projections are transiently associated with broad bands of postsynaptic ACh receptor clusters. Schwann cell precursors, that initially accompany peripheral projections, are absent from axonal terminations within muscle. Following an aborted attempt at synapse formation, sensory and motor nerves withdraw and eventually degenerate. Our data demonstrate the essential role of CRD-NRG-1 mediated signaling for coordinating nerve, target, and Schwann cell interactions in the formation and maintenance of peripheral synapses.

Alt rnative splicing generates at least fifteen different *neuregulin-1* (NRG-1) cDNAs. [1,6] All identified NRG-1 isoforms can be broadly classified into three mutually exclusive categories: Type I and II isoforms that contain an Ig-like domain (Ig-NRGs) and Type III isoforms that contain a cysteine-rich domain (CRD-NRGs) N-terminal to a common EGF-like sequence (Fig. 1A). [3,6-11] Although different NRG-1 isoforms show distinct patterns of spatial and temporal expression during embryogenesis (data not shown), it is not clear to what extent these isoforms mediate distinct biological responses. [3,11] Mice homozygous for disruptions of all NRG-1 isoforms, all Ig-NRG-1 isoforms, or all isoforms containing a cytoplasmic tail die at E10.5 from heart defects, prior to significant expression of CRD-NRG-1 isoforms. [12-15] As such, these mice provide no direct evidence as to the possible functions of CRD-NRG-1 isoforms in neural development or synaptogenesis.

CRD-NRG-1 isoforms are the predominant NRG-1 transcripts in the murine nervous system after E10.5. In particular, the temporal pattern of CRD-NRG-1 expression in hindbrain and spinal cord visceral and somatic motor neurons, as well as in cranial and trunk sensory ganglia, suggests an important role for this isoform in synapse formation (Fig. 1E, b & data not shown). [3,5,10,11] To assess the potential role of CRD-NRG-1 isoforms in neural development, we generated mice selectively lacking all NRG-1 isoforms that contain a CRD domain within the N-terminus.

CRD-NRG-1^{+/-} embryonic stem (ES) cells were isolated following homologous recombination with a mutated CRD-encoding exon that included a nonsense mutation/frameshift and a novel XbaI site inserted into the codon immediately 3' of the last methionine (Fig. 1B). These ES cells were used to generate germline chimeras following injection into mouse blastocysts. Male chimeras transmitting the mutant allele were used to produce fertile heterozygote offspring.

CRD-NRG-1$^{(+/-)}$ mice were interbred to produce homozygote CRD-NRG-1$^{(-/-)}$ mice. In the resulting litters. cleaned. dead pups were consistently found among viable, healthy littermates (Fig. 1E. a). Genotype analysis identified the dead pups as CRD-NRG-1$^{(-/-)}$ (22% of first 132 P0 pups) (Fig. 1C). As expected. mutant animals expressed both Ig-containing and mutant CRD-containing mRNAs (Fig. 1D). *In situ* hybridization revealed that the spatial and temporal expression pattern of the nonsense mutation containing-CRD-NRG-1 mRNA was not altered in the mutants (Fig. 1E. b vs. b', & data not shown).

.CRD-NRG-1$^{(-/-)}$ pups were limp, and limb movement was not detectable (Fig. 1E. a). These pups did not breathe. rapidly became cyanotic, and finally were unresponsive to all stimuli despite the presence of a heartbeat for several minutes after birth. Histological analysis confirmed that hearts of CRD-NRG-1$^{(-/-)}$ mice were normal but that the lung alveoli failed to expand. confirming that CRD-NRG-1$^{(-/-)}$ pups never take a breath (data not shown and Fig.1E. c *vs.* c').

Examination of P0 mutant diaphragms revealed only remnants of nerve-muscle synapses (Fig. 2A. a *vs.* a'). Mutants had decreased skeletal muscle mass. and mutant skeletal muscle had central nuclei with prominent nucleoli compared to the elongated. eccentric. peripheral nuclei in control muscle (Fig. 2A. d vs. d' and data not shown). CRD-NRG-1$^{(-/-)}$ mice lacked an intra-diaphragmatic phrenic nerve plexus (Fig. 2A. a *vs.* a'). Bands of α-bungarotoxin (αBgTx) positive acetylcholine receptor (AChR) clusters. reminiscent of end-plate zones. were present but abnormally broad (~6 times wider than littermate controls) (Fig. 2A. b *vs.* b'). These diffuse bands of AChR clusters. the absence of associated nerve. and the scattered neurofilament(+) fragments indicate prior presence. and subsequent degeneration. of motor projections. Indeed. examination of diaphragms from E14.5. E15.5. E18.5, and P0 mutant mice revealed

elaborate phrenic nerve arborization at E14.5 and progressive loss of these nerve fibers during subsequent stages of development (Fig. 2B, f vs. f', and data not shown). Phrenic nerve processes in E14.5 CRD-NRG-1^{-/-} mice were grossly abnormal with extensive defasciculation, aberrant axonal branching patterns, and projections extending as far as the most lateral borders of the diaphragm (Fig. 2B, f vs. f'). Although post-synaptic AChR clusters were sometimes detected subjacent to neurofilament(+) and synaptophysin(+) structures in mutant E14.5 diaphragms, these clusters were typically more diffuse and more often devoid of associated nerve than age-matched controls (Fig. 2B, g vs g').

A similar pattern of defective nerve-muscle interaction was detected in intercostal and limb muscle groups (e.g., scattered αBgTx(+)-AChR clusters and lack of co-localization with nerve; Fig 2B, e vs. e' and data not shown). Acetylcholinesterase (AChE), which is normally enriched at the site of nerve-muscle contact following the establishment of synapses, was scattered and faint in mutant muscle (Fig. 2A, c vs. c').[16] Thus, in CRD-NRG-1^{-/-} mice, motor axons initially extend to and contact the target muscle and some aspects of pre- and post-synaptic specializations were evident. However, as these preliminary synaptic interactions were not sustained, we next examined if the number of motor neurons was affected.

Somatic motor neurons were visualized by in situ hybridization to the vesicular ACh transporter (VAT) mRNA.[17] The number of VAT(+) cell bodies within the ventral horn of C1-C6 of E18.5 mutants was 60% less than that of controls (Fig. 3A, a vs. a', b). A dramatic reduction in the diameter of peripheral nerves arising from motor pools at other spinal cord levels, including those within intercostal and hindlimb muscles, was also evident in E18.5 CRD-NRG-1^{-/-} animals compared to controls (Fig. 2B, e vs. e' and data not shown). Likewise, E18.5, but not E14.5, cervical dorsal root ganglia and their

peripheral sensory projections were dramatically decreased in size in CRD-NRG-1[(-/-)] compared with control animals (Fig. 3B, d vs. d' and data not shown). The observed degeneration of peripheral sensory and motor nerve projections by E18.5 is likely to account for the abnormal morphology of forelimbs and absence of all limb movement, evident in both E18.5 and P0 CRD-NRG-1[(-/-)] mice (Fig. 1E, a and data not shown).

We next examined the development of the sensory and motor projections prior to arrival in their respective target fields. At E11, whole mount immunostaining of mutant embryos with β-Tubulin III, a pan-neuronal marker, revealed that initial projections of both sensory and motor neurons were fasciculated (Fig. 4B, h vs. h').[18] At later stages and at more caudal levels of mutant embryos, these terminal projections displayed dramatic defasciculation, aberrant and irregular branching, and markedly increased terminal sprouting (Fig. 3B, f vs. f'). Likewise, in caudal hindlimb muscle of older mutant animals, supernumerary terminal motor sprouts were visualized with VAT Ab staining (Fig. 3B, g vs. g'). The VAT(+) sprouts and AChR clusters, visualized as αBgTx "hot spots," were each detected without the complementary marker of nerve-muscle synapse formation (data not shown). Finally, at later times and at more rostral levels of mutant embryos, the terminal projections of sensory and motor neurons were smaller, bulb-shaped, and compact, when compared to the more splayed nerve endings in control animals (Fig. 3A, c vs c'; 3B, e vs e').

Taken together, these data indicate a temporal and rostral-caudal pattern whereby the initial trajectory and outgrowth of peripheral nerves in CRD-NRG-1[(-/-)] mice are grossly normal. However, once within the terminal fields, projections defasciculate, displaying profuse and aberrant branching, and subsequently, appear bulbous and then fragmented, consistent with their ultimate retraction and degeneration.

P ripheral nerves projecting to their targets are lined by neural crest-derived

Schwann cell precursors that express erbB3. p75 NGF receptor. and S100β.[19,20,21] At

E18.5. mutant embryos lacked erbB3/p75-expressing cells in both dorsal and ventral

roots (Fig. 3B, d vs. d' and data not shown). Likewise. S100β expressing cells were

absent from intramuscular branches of the phrenic nerve in E14.5 mutant diaphragms

(Fig. 2B. h vs h'). Although erbB3(+) cells were detected along nerve roots in E11

mutant mice, the progressive. rostral-caudal loss of these cells was evident by E12 (Fig.

3B, h vs. h'; Fig. 4B. j vs. j'). The spatial pattern of Schwann cell precursor loss was

revealed by examination of more caudal structures in older CRD-NRG-1^{-/-} animals. In

peripheral hindlimb muscle of E18.5 CRD-NRG-1^{-/-} mice. S100β(+) cells lined the

VAT(+) axons and preterminal branches but were not detected at the site of VAT (+)

nerve terminations (Fig. 3B. l). In contrast, in control embryos VAT(+) preterminal nerve

branches and nerve terminations were always associated with S100β (+) staining (Fig.

3B, l').

Cranial sensory ganglia strongly express CRD-NRG-1 isoforms (Fig. 1E. b: Fig.

4A. a. b. and data not shown) CRD-NRG-1. erbB3 and erbB4 mRNA have been

detected in the peripheral targets of these ganglia. including vibrissa follicles (Fig 4A. c-

e).[11] The fate of these neuronal populations and their associated erbB3(+) Schwann

cells was significantly altered in CRD-NRG-1^{-/-} mice. Both neural crest and otic

placode-derived cellular elements of cranial ganglia appear to form in CRD-NRG-1^{-/-}

mice. but their initial projections were markedly less robust in mutants (Fig. 4B. h vs. h').

As cranial nerves enter their target fields. the pattern of nerve defasciculation and

extensive branching was seen (Fig. 4B. h vs. h'). The subsequent demise of cranial

nerve projections was apparent by E12.5. and by E14.5. cranial ganglia (including the

trigeminal and geniculate) and their associated nerves were dramatically reduced in size in CRD-NRG-1(-/-) mice (Fig. 4B. i vs. i' and data not shown). Furthermore. cranial nerve projections were nearly devoid of associated Schwann cell precursors in CRD-NRG-1(-/-) mice compared with controls (Fig. 4B. i vs. i' and data not shown). Examination of cranial nerves at earlier stages of development reveals the initial appearance and subsequent loss of erbB3(+) cells along the ascending spinal accessory (N11n) nerve and the coalesence of vagus nerve (N10n) and in the ganglia and along the peripheral projections of the trigeminal (N5g), geniculate (N7g), glossopharyngeal (N9g), and vagal (N10g) ganglia (Fig. 4B. j vs. j' and data not shown).

CRD-NRG-1 is expressed in VAT(+) cells of cranial motor nuclei. such as the trigeminal (N5N), facial (N7N). and dorsal motor nucleus of the vagus (Fig. 4A, f & g and data not shown). At E18.5 the number and distribution of VAT(+) neurons within the brainstem of CRD-NRG-1⁻ embryos was abnormal with the most striking changes seen in the motor nuclei of cranial nerves N5N and N7N (Fig. 4B. k vs. k'; i vs. i'). Preliminary analysis of other VAT(+) neurons within brainstem and forebrain nuclei also indicates possible changes in development and synaptogenesis (D.W. and L.W.R.. in preparation).

In summary, CRD-NRG-1 is an essential. nerve-derived component of peripheral synaptogenesis. The phenotype associated with the selective disruption of CRD-NRG-1 mediated signaling is distinct from all other NRG-1 ligand or receptor knockouts reported to date. CRD-NRG-1⁻ mice die within minutes of birth, unable to breathe due to the lack of functional phrenic nerve-diaphragm synapses. The defects in CRD-NRG-1⁻ mice are evident as a temporal progression and in a rostral-caudal pattern during embryogenesis. Thus. both motor and sensory nerve projections initially emanate from

hindbrain to lumbar spinal cord levels. However, with the arrival of projections within

their respective targets. mutant peripheral nerves branch extensively, and Schwann cell

precursors, initially associated with peripheral projections. do not survive. By parturition.

CRD-NRG-1[(-/-)] mice have severe deficits in the number and extent of motor and sensory

neurons, consistent with extensive neural degeneration. At birth, skeletal muscle targets

display features of immaturity similar to those found in human myotubular myopathy.[22]

Genetic defects in a muscle phosphatase have been implicated in the severe X-linked

form of this disease.[23]

.NRG-1 signaling has long been implicated as an important component for

survival and differentiation during Schwann cell development.[2,13,19] Loss of Schwann cell

precursors is a phenotype shared by CRD-NRG-1[(-/-)] and erbB3 null mice.[21] The early

effects of postnatal axotomy on Schwann cell apoptosis are partially reversed by

exogenous NRG-1 protein.[20,24] Furthermore, previous studies comparing NRG-1[(-/-)] mice

and Ig-NRG-1[(-/-)] mice led to the proposal that non-Ig containing NRG isoforms might be

required for Schwann cell survival.["] The unique neural specificity of CRD-NRG-1

expression in mouse and the progressive loss of erbB3(+) Schwann cell precursors

along peripheral nerve projections in CRD-NRG-1[---] mice implicate CRD-NRG-1

isoforms as the essential, nerve-derived trophic signal for erbB3-mediated Schwann cell

survival.

Sensory and motor neurons are critically dependent on target-derived trophic

support during early development.[25,26] Likewise. the development and terminal

differentiation of target muscle requires interaction with motor nerve. The phenotype of

CRD-NRG-1[(-/-)] mice demonstrates that CRD-NRG-1 isoforms are necessary

components comprising the reciprocal cascades of these cellular interactions. Birth,

initial differentiation and peripheral projections of motor and sensory neurons proceed

apace in the absence of CRD-NRG-1 isoforms. Initial aspects of post-synaptic organization, such as clustering of AChRs in muscle. are also detected in CRD-NRG-1^{-/-} mice. The AChR-cluster inducing signal. agrin. acts through MuSK receptor complexes in skeletal muscle. [29,30] It is noteworthy that mice in which these genes are disrupted show abnormalities in neuromuscular junction formation. but motor projections neither withdraw nor degenerate. [29,30]

In contrast, the genetic disruption of CRD-NRG-1 results in both pre- and post-synaptic defects consistent with loss of reciprocal. inter-dependent nerve and target-derived trophic support. Furthermore. the target-derived support that appears to be required for the persistence of the incoming nerves is not constitutively available but must be elicited by a nerve-derived signal. We propose that this nerve-derived signal is CRD-NRG-1. As Schwann cell precursors might also provide essential trophic support for developing nerves, and these cells fail to survive in CRD-NRG-1^{-/-} mice. it is possible that Schwann cell precursors and/or peripheral targets respond individually or synergistically to a common nerve-derived. CRD-NRG-1-mediated signal that normally elicits context-dependent. trophic and synaptogenic cues.

METHODS

Generation of CRD-/- Mice. A mouse 129/SV genomic library (Lamba EMBL3. Stratagene) was screened with a mouse CRD-specific probe. Positive clones were mapped and sequenced revealing that the CRD-domain is encoded by a single exon. PCR-mediated site-directed mutagenesis and standard protocols in molecular biology were used to create the targeting vector. ES cells were electroporated, selected for G418 resistance (150 µg/ml active substance), and screened by Southern blot for homologous recombination. ES clones. in which homologous recombination resulted in replacement of one of the wild-type NRG-1 alleles with the mutant gene. were injected in C57/BL6/J blastocysts. Chimeric males were mated with 129/Sv females to generate heterozygotes on a pure 129/Sv background.

RT-PCR. Total brain/spinal cord RNA was isolated using Trizol (Gibco BRL) following the manufacturer's protocol. and first-strand cDNA was made using SuperScript II reverse transcriptase following the manufacturer's protocol (Gibco BRL). Mouse-specific primers and oligonucleotide probes corresponded to the following: from Genbank L41827. CRD 5' primer (549-565). CRD-probe (582-605); from Genbank u02318: Ig 5' primer (499-518), Ig/CRD 3' primer (885-903), egf-cyt primers (913-931), (1184-1203). Ig probe (608-631), TM probe (1080-1103). Note that all primer pairs span exon-intron boundaries.

In Situ Hybridization and Cell Counts. In situ hybridization (ISH) probes were as follows: mouse probes specific for CRD domain (corresponding to Genbank AF045654. 607-1206). PAN-NRG-1 (corresponding to Genbank AF045654. 867-1277), erbB3

(corresponding to Genbank u29339.3321-4113), erbB4 (corresponding to Genbank L07868. 3088-3957), VAT (gift of Dr. J. Dedman: Genbank AF019045. 945-2157) or rat p75 NGF receptor (gift of Dr. Moses Chao). Embryos were fixed in 4% paraformaldehyde by transcardial perfusion, followed by post-fixation overnight at 4°C. Following cryoprotection in 30% sucrose. embryos were embedded in OCT and 12 μm sections were cut on a cryostat. ISH was done as previously described.[31] For cell counts. 10 μm transverse serial sections were cut through C1-C6 and stained for VAT. Total number of nuclei of VAT(+) cells in every sixth section was then counted. Counts between ages are not strictly comparable as total cell numbers were not corrected for split or multiple nucleoli. We feel our counts reflect a conservative estimate of the cell loss, as motor neurons with retracted processes are included. Whole-mount ISH was done as previously described.[32]

Histological Analysis and Immunostaining. Lung was fixed overnight in 4% paraformaldehyde and embedded in paraffin. 8μm sections were cut and stained with H&E. Diaphragm was sandwiched between liver. flash-frozen in liquid-nitrogen cooled isopentane. and 6 μm sections were stained with H&E. AChE staining was done as described previously.[33] Whole-mount immunostaining using monoclonal antibody to β-Tubulin III (Sigma) was done as previously described.[34] After staining, embryos were dehydrated and cleared in cedarwood oil. Whole-mount diaphragm staining was done as previously described.[29][30] Immunofluorescence labeling employed monoclonal anti-neurofilament NF-60 & 168 antibodies (Sigma). anti-S100β antibody (DAKO), FITC-conjugated alpha btx (Molecular Probes). anti-synaptophysin antibody (gift of Dr. Pietro de Camilli). and rhodamine or FITC-conjugated anti-mouse or anti-rabbit antibodies (Jackson Immunoresearch)

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Figure 1 Disruption of CRD-NRG-1 gene products.

(A) Schematic diagram of β-form *neuregulin-1* gene products (.β-forms differ from α-forms in the EGF-like domain). Splice variants of the NRG-1 gene and the various nomenclatures used in the literature are shown. a,b,c refer to three variants of the cytoplasmic domain. Ext, extracellular. Tm, transmembrane, CYTO, cytoplasm.

(B) Schematic diagram of the wild-type CRD-containing allele, the targeting vector, and the mutated allele. The barbell indicates the site of the nonsense mutation/Xba I site. Arrow and circled X schematically indicate that the nonsense mutation creates a translation stop codon. Note that the *neo* cassette is inserted in the antisense orientation into the intron 3' to the CRD-exon.

(C) Southern blot analysis of genomic DNA. Left,. EcoRI/Eco RV double digest. with probe A; Right, Xba I digest. with probe B.

(D) Southern Blot analysis of cDNA to examine neonatal *neuregulin-1* transcripts. Top. Schematic representation of CRD and Ig containing *neuregulin-1* cDNAs. Arrowheads are pcr primers: solid bands are Ig and TM oligo probes used for Southerns: hatched area is CRD-specific oligo probe. Note the presence of CRD-containing, Ig-containing, and EGF-TM-cytoplasmic containing transcripts in CRD-NRG-1$^{+/+}$ and CRD-NRG-1^{--} mice and that CRD transcripts are digested with Xba I only in CRD-NRG-1^{--} mice.

(E) a Newborn CRD-NRG-1^{--} mice lack spontaneous movement and are cyanotic. **b,b'** Whole-mount in situ hybridization with CRD-specific probe of EII CRD-NRG-1$^{(+/-) \& (-/-)}$ mice. N5g, trigeminal: N10g, vagal ganglia: sc. spinal cord: drg, dorsal root ganglia. **c,c'** Paraffin-section of P0 lung stained with H&E. **(c)** asterisks indicate expanded alveoli in CRD-NRG-1^{--} mice. **(c')** carets point to collapsed airspaces in CRD-NRG-1$^{-/-}$ mice.

Figure 2 Defective Neuromuscular Synaptogenesis in CRD-NRG-1⁻ mice. (A) **a,a'** Immunofluorescence labeling (100X) of P0 diaphragm with anti-neurofilament antibodies (red) or with **b,b'** anti-neurofilament antibodies (red) & α-bungarotoxin (αBgTx) (green). (a') arrows point to remnant neurofilament(+) staining in mutants. carets (b) and arrow (b') point to colocalization of neurofilament and αBgTx staining. **c,c'** Acetylcholinesterase staining (AChE) (400X) of P0 diaphragm. (c) carets point to strong, aligned AChE staining in controls. (c') arrows point to weak, scattered AChE staining in mutant. **d,d'** H&E staining of fresh-frozen P0 diaphragm muscle. (d) carets indicate eccentric. peripheral nuclei. (d') arrows point to central nuclei. (B) **f,f'** Immunofluorescence with anti-neurofilament antibodies (red); **e,e',g,g'** anti-neurofilament and anti-synaptophysin antibodies (red) & αBgTx (green); **h,h'** anti-neurofilament antibodies (green) & anti-S100β antibody (red). (e,e') E18.5 intercostal muscle (400X), (e) asterisks point out overlap of neurofilament and αBgTx, (e') arrows indicate examples where staining does not overlap. **f,f'** E14.5 diaphragm (100X). (f) asterisks indicate normal branching pattern of phrenic nerve and termination of intradiaphragmatic branches in central region of muscle. (f') arrows highlight defasciculated axons with irregular branching patterns. repeated neurite crossings, and aberrant extension to the most lateral edges of the diaphragm.. **g,g'** E14.5 diaphragm (400X). (g) asterisks indicate the organized endplate zone where nerve terminals overlie clusters of αBgTx-labeled AChRs. (g') arrows indicate examples where synaptophysin & αBgTx do and do not overlap. Note disorganized endplate zone with nerve terminals approaching AChRs from many directions and some nerve terminals passing over αBgTx staining. **h,h'** E14.5 diaphragm (400X). arrows in (h) indicate S100β(+) cells along peripheral nerves absent in (h')

Figure 3 Abnormal projections and subsequent degeneration of peripheral nerves in CRD-NRG-1⁻⁻ mice. **(A)** a,a' *In situ* hybridization with vesicular ACh transporter (VAT)-specific probe of 10 μm sections through cervical enlargement of E18.5 spinal cord (200X). **b** Number of motor neurons present in spinal cord levels C1-C6 in control and mutant embryos (n=3 for each age and genotype). **c,c'** Whole-mount immunostaining of E12.5 limb with β-Tubulin III antibody (70X). **(c)** black and yellow asterisks show splayed nerve terminations of spinal nerves and cutaneous sensory projections in controls, respectively. **(c')** black and yellow arrows indicate bulbous. compact terminations of spinal nerves and cutaneous sensory projections in CRD-NRG-1⁻⁻ mice, respectively. **(B) d,d'** *In situ* hybridization with erbB3-specific probe of 10 μm sections through cervical enlargement of E18.5 spinal cord (200X). **d** asterisks indicate sensory root lined with erbB3(+) cells. **d'** arrows point to the sensory root that is devoid of erbB3(+) cells. **e,e',f,f'** Whole-mount immunostaining of E12.5 embryos with β-Tubulin III antibody (70X); lmb, limb. **(e,e')** asterisks and arrows indicate the cervical sensory cutaneous projections. **(f,f')** asterisks and arrows point to trunk lateral cutaneous sensory branches. **g,g'** Immunofluorescence with anti-VAT antibodies of E18.5 hindlimb muscle (green) (200X). Asterisks **(g)** & arrows **(g')** arrows point to nerve terminals. **h,h'** Whole-mount *in situ* hybridization of E12 embryos with erbB3-specific probe (70X). **(h)** asterisks point to erb3(+) staining along peripheral nerves emanating from caudal spinal cord levels. **(h')** arrows point to peripheral nerves which show decreased erbB3(+) staining. Note that embryo in **(h')** is older than embryo in **(h)**. **i,i'** Immunofluorescence with anti-neurofilament antibodies (green) & anti-S100β antibody (red) (400X). **(i)** asterisks indicate colocalization of neurofilament & S100β staining in nerve terminations of control. **(i')** yellow arrows point to colocalization of neurofilament and S100β along preterminal branches; white arrows point to nerve terminations devoid of S100β staining.

Figure 4 Hindbrain Deficits and Loss of Schwann cell precursors in CRD-NRG-1⁻ mice .

(A) a-b *In situ* hybridization of 10 µm transverse serial sections of trigeminal ganglion of E14.5 embryos. **(a)** CRD-NRG-1-specific probe (100X). **(b)** Pan-NRG-1-specific probe (100X). **c-e** *In situ* hybridization of 10 µm coronal serial sections of vibrissa follicles of E18.5 embryos. **(c)** CRD-specific probe (200X); **(d)** erbB3-specific probe (200X); **(e)** erbB4-specific probe (200X). **f,g** *In situ* hybridization of 10 µm coronal sections of E18.5 embryos with CRD-NRG-1-specific probe. **(f)** trigeminal motor nucleus (200X) **(g)** facial motor nucleus (200X). **(B) h, h'** Whole-mount immunostaining of E11 embryos (70X) with anti-β-tubulin III antibody. **(h')** Arrows indicate the onset of abnormal branching of cranial nerve projections N5n_{man} & N7n. N5g, trigeminal; N7g/N8g, vestibulo-cochlear; N9g, petrosal; N10g, nodose ganglia; op, opthalmic, max, maxillary, man, mandibular branches of N5n, trigeminal nerve; N7n, facial; N10n, vagus; N11n, spinal accessory; N12n, hypoglossal nerve; C1,C2 cervical dorsal root ganglion and cervical spinal cord projections. **i,i'** *In situ* hybridization with erbB3-specific probe of trigeminal ganglion of E14.5 embryos. **(i)** asterisks indicate peripheral projections lined with erbB3(+) cells. T-bar indicates the width of nerve (100X). **(i')** arrows point to the peripheral nerve projection in mutants (100X). **(j,j')** Whole-mount *in situ* hybridization with erbB3-specific probe of E12 embryos (70X). **(j)** black and green arrows point to erbB3-positive cells along N11n and its convergence with N10n and along peripheral nerve projections of cervical dorsal root ganglia, respectively. White arrows point to erbB3(+) positive cells in developing muscle. **(j')** black and green arrows point to the dramatic loss of erbB3(+)-staining along N11n, the coalescence of N10n, and along the projections of cervical dorsal root ganglia, respectively. White arrowhead points to the remaining erbB3 staining in developing muscle in CRD-NRG-1⁻ mice. **k,k',l,l'** *In situ*

hybridization of E18.5 coronal sections through hindbrain with a vesicular ACh transporter (VAT)-specific probe. Note the reduction in size of facial motor nucleus (50X) **(k vs k')** and of trigeminal motor nucleus (200X) **(l vs l')** and abnormal cell migration (arrows, **k'**) in CRD-NRG-1$^{-/-}$ mice. Aq, aqueduct of IVth ventricle, m, midline, LDT, lateral dorsal tegmenal nucleus; PBr, parabrachial nucleus; N6n, abducens nucleus; N7n, facial nucleus. Dotted line in **k,k'** indicates the midline, m.

The present invention provides an assay for the determining the amount of her 2 receptor in a bodily fluid from a subject and an assay for other heregulin receptors which comprises a) obtaining a bodily fluid from a subject or a

5   sample from a subject (i.e. a brain punch biopsy, blood, serum, plasma, urine, etc.); b) contacting the sample with cells which are transfected with a reporter construct (the construct being CRD-neuregulin with a cytoplasmic tail which has a detectable marker attached to it); c) measuring the

10   detectable label in the nucleus of the cells and thereby determining the amount of her 2 receptor in the bodily fluid from the sample.

The neuregulin in the bodily fluid binds to a receptor on

15   the surface of the transfected cell and there will be cleavage of the external domain and then cleavage of the internal cytoplasmic domain. The cytoplasmic domain will then be translocated into the nucleus and the detectable label will be available for detection in the nucleus. The

20   detectable label may be green fluorescent protein or VP16.

Another assay which is provided for in the present invention is an assay which comprises obtaining a brain punch from a subject and performing immunohistochemistry on frozen

25   sections or preserved sections of the tissue obtained using a detectably labeled antibody specific for the cytoplasmic domain of neuregulin and determining the percentage of nuclei that are showing label in the tissue sample.

30   The nuclear targeting domain of neuregulin may be found in Yang et al. and begins at amino acid number 230 (KTKKQRKK).

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